

Nature's new venture in Japan

Nature will now be printed in Japan as well as in Britain and the United States. One objective is to give readers a better service. Another is to draw the scientific community of Japan more into the general swim.

ANY international publisher will say it is ridiculous that a journal with a tiny circulation (just over 34,000 at the end of May, probably 1,000 greater by now) should ape the *Times* and *Newsweeks* of this world by printing replicas of itself in Japan. Are not two printing centres already a sufficient headache for such an esoteric publication, especially when most other science journals appear content with only one? That is what the sober-sides will say. *Nature* has rejected that opinion on the simple grounds that a journal with international pretensions cannot entrust the timeliness of its distribution around the world to the habits of local postal services here and there. Journal publication is a socially valuable activity when it gives readers the sense of community that springs from knowing that the same information is more or less simultaneously available, and is thus diminished by delay. *Nature* intends that it should be delivered to addresses in Japan within a few days of its formal publication in London and that its cost to private Japanese subscribers shall be enormously reduced, by something like 60 per cent.

Many also know that Japan is the cockpit of social and industrial change in East Asia, itself probably next century's powerhouse. *Nature's* concern to be quickly available in Tokyo must therefore be recognized as being alloyed with considerations of where its future lies.

Does that mean that *Nature* now believes that the future of fundamental science is in the hands of a people so willing to impoverish its academics as to make Mrs Thatcher's British academic dependants seem like princes, so able to educate its young that most of them are literate in the world's most difficult language and knowledgeable in other things as well, yet so eager for the quick buck that their licensed companies will sell off allies' military secrets without thinking twice (last week's Toshiba scandal refers)? It would, of course, be foolish to attempt to guess which centres will emerge as the most productive sources of innovation. Most probably the pattern of scientific work will be more widely distributed, and more dependent on international collaboration, than in the recent past.

But the Japanese people, who have made distinguished contributions to fundamental science over the past half-century, are now sufficiently challenged by the probably mistaken self-diagnosis of themselves as being strong on ingenuity but short on creativity as to wish to change that state of affairs. There are many who will laugh at the idea that the world's most successful industrial power should be cudgelling its brains to find ways of being more creative. It will nevertheless be a great surprise if Japan has not so successfully built on its quickly growing roster of excellent research centres that it soon becomes as powerful in basic science as it is already in the application of science.

The Japanese government's enthusiasm, at least until a few months ago, for its Human Frontiers programme (see page 100) is one index of its eagerness to find new ways of doing scientific business. Critics of the project will say that the project is a recipe for making vagueness respectable, but that is to mistake what Japan has been attempting. The objective has been to see whether new technology will spring from biology in the century ahead, as it has sprung from physical science in the century past. The government has gone trawling for ideas internationally, no

doubt hoping that these consultations would also engender enthusiasm among potential partners in time for that to have been reflected in last month's economic summit meeting at Venice. That calculation has been disappointed, partly because Japan neglected to play the old game of putting money on the table. But the project, it must be hoped, is not yet dead. Putative partners in the West should use this opportunity to discover what Japan is looking for, lest the opportunity should be foreclosed. *Nature* will do more to find out, and will report.

Meanwhile, there are more immediate considerations that will ensure that printing *Nature* in Japan will be entertaining and rewarding not merely for *Nature* and its Japanese readers but for people elsewhere. One hope is that, by means of a stronger presence of *Nature* in Japan, Japanese researchers will more readily overcome their diffidence at publishing in the international literature. Another is that Japan will be a conduit to and for other parts of east Asia, China in particular. Meanwhile, it should be made widely known that, in making arrangements to print in Japan, *Nature* has encountered none of the legendary non-tariff barriers to trade by means of which Japan is said to keep itself to itself. (It may have helped incalculably that, in this enterprise, *Nature* has had the assistance of its owners, the international publishing group Macmillan, which is particularly strong in what Occidentals still perversely call the Far East.) But the Japanese people concerned have been, as always, hospitable, helpful and exceedingly efficient. What else does one now expect? □

Going back to GO

The plight of the Geological Survey shows what is wrong with British research administration.

ONCE upon a time (in 1835), there sprang into being a happy and productive research organization, called the Geological Survey (later rechristened the Geological Survey of Great Britain and Ireland, afterwards of the United Kingdom), that sent men and women also into the field with hammers, and which produced delicately coloured maps that were nevertheless of great utility. Productive contentment reigned until the early 1960s, when the Geological Survey had the misfortune to fall upon good times: it was rechristened yet again as the Institute of Geological Sciences, and its staff was multiplied threefold. Its then director, Sir Kingsley Dunham, an unreflective man so powerful that he could not suffer even the wise gladly, malignly enjoyed being the tail that wagged the dog of the then newly created Natural Environment Research Council (NERC). Then, after Dunham's time, NERC had its own back, saying that survey work would have to be paid for by those who found it useful. The Butler committee which has been brooding about the condition of the British Geological Survey (see page 102) now recommends going back to GO. It is right to do so. Even well-worked countries such as Britain need geological survey organizations. The pity is that so much blood has been spilled in reaching this conclusion. Mrs Margaret Thatcher, the British Prime Minister, will be furious if she gets to know what has been going on. □



Storm brewing over Toshiba's illegal exports

- Company president resigns over ban
- Soviet 'silent submarine' theory

Washington & Tokyo

US ANGER over the illegal export of advanced milling machinery to the Soviet Union by a Japanese and a Norwegian company continues to mount some two months after the incident first became public.

Last week, a Senate trade bill was amended so as to ban for between two and five years the import into the United States of products made by the two companies involved — Toshiba Corporation of Japan and Kongsberg Vaapenfabrikk of Norway. Within hours, both the chairman and the president of Toshiba had resigned their posts. The Senate bill, as amended, would seek to recover damages from the two companies.

The milling machines may have enabled the Soviet Union to build submarine propellers whose noise emission is a minimum and which are therefore relatively immune from US listening equipment. US sources say that millions of dollars may now have to be spent on the improvement of listening equipment.

The illegal deal was initiated in 1980, when a Soviet trade organization persuaded Toshiba Machine Company, a majority-owned subsidiary of Toshiba Corporation, to provide automated propeller manufacturing equipment. To avoid attracting attention to the sale, Toshiba Machine insisted on using a standard export broker and enlisting the aid of the Norwegian company. Under the terms of a contract signed in Moscow in 1981, Toshiba agreed to supply four milling machines and Kongsberg Vaapenfabrikk the associated computers and software.

It seems agreed that the companies well knew that export of the machines was prohibited under regulations of the inter-governmental coordinating committee on trade in sensitive equipment (COCOM). False documents were prepared to conceal the true nature of the equipment and which named a civilian end user. The machines were delivered to a Soviet Navy propeller production facility in Leninrad; installation was completed in 1984.

The US Department of Defense says that, in 1986, its underwater sonar detectors began to pick up silent-propeller Soviet submarines not detected by passive listening devices. The illegal sale of the equipment was first discovered by the United States in the subsequent investigation of improvement of Soviet propeller technology. It has since emerged that

Japan's Ministry of International Trade and Industry (MITI) had heard of the deal at an earlier stage, when a former employee of a Japanese company that had acted as an intermediary went to officials at COCOM's Paris headquarters. But MITI failed to conduct a full investigation.

The exported machinery is capable of milling extremely smooth complex surfaces on very large propellers, but it has not been proven that the new Soviet propellers were actually produced on the illegally exported machines. Japanese sources point out that the leak of information on submarine detector equipment by a US spy ring must have been equally important in helping build submarines the United States would find hard to detect.

Japan's Prime Minister Yasuhiro Nakasone has tried to make amends by offering to help the United States to develop anti-submarine technology. But this possibility, brought up during the recent visit of Defense Secretary Caspar Weinberger to Tokyo and to be followed up by a visit from US experts, has not been seen as sufficient in the US Senate. Nor has the Japanese government's ban on Toshiba exports to the Soviet Union for the year ahead, which was referred to by Senator Jake Garn (Republican, Utah) as a "slap with a wet noodle".

The resignation of the two senior officials of the Toshiba Corporation was accompanied by public apologies for "straining US-Japan relations" and for the "consequences of Toshiba Machine's behaviour for the security of the Western world". Despite the nobility of their public act, however, the real feelings of the company officials may be somewhat different. Toshiba officials continue to stress that the parent company held only 50.8 per cent of Toshiba Machine's stock and, as is usual in Japan, did not monitor in detail what went on in the subsidiary.

To punish the parent for a grown-up son's behaviour is seen as unfair. A US accounting company is to examine Toshiba Corp.'s books to certify that it knew nothing of the illegal sale.

This action, and the resignations, are unlikely to affect the demands for action in Washington. Although an attempt to match the Senate amendment with an amendment to a bill in the House of Representatives failed on a point of order, legislation will be considered in coming weeks.

Alun Anderson & David Swinbanks

Superconductor success for Japanese

Tokyo

JAPAN and the United States are racing neck and neck in the development of high-temperature superconductors. It is less than two months since IBM scientists announced success in growing a single-crystal thin film with a current-carrying capacity greater than $100,000 \text{ A cm}^{-2}$ at the temperature of liquid nitrogen (*Nature* 327, 89; 1987). Researchers at Nippon Telephone and Telegraph (NTT) have now topped that record by a factor of more than ten by using essentially the same technique.

One of the major barriers to development of electronic devices from the new superconductors is the low current-carrying capacity of the oxide ceramics. But this barrier is fast being eroded.

Last week (30 June) NTT announced the development of a thin film of yttrium-barium-copper oxide with a maximum critical current density of 1.8 million A cm^{-2} at 77.3 K. The single-crystal film was prepared by RF magnetron sputtering on a monocrystal SrTiO_3 substrate.

As in the case of the IBM results, the critical current is dependent on direction in the crystal: the maximum current was observed parallel to the a/b plane of the copper oxide, while in the c -axis direction, parallel to the surface of the film, the critical current density was two orders of magnitude lower at $10,000 \text{ A cm}^{-2}$.

Almost simultaneously with the NTT announcement, Sumitomo Electric Industries Ltd, a major cable manufacturer, claimed to have developed a bulk ceramic which is superconducting at room temperature. The ceramic, composed of yttrium-barium-copper oxide and a "secret ingredient", was compressed into five 3-mm-thick disks (diameter 7 mm) which, depending on the disk, showed zero resistance at temperatures ranging from 77 to 300 K. One of the disks (unspecified) "seemed" to show the Meissner effect, according to Sumitomo. But the superconducting properties in the material were unstable and only lasted from one to seven days. Unequivocal evidence of room-temperature superconductivity has yet to be found.

David Swinbanks

Nature's Tokyo print

FROM this week's issue, *Nature* will be printed regularly in Tokyo, for distribution in Japan and elsewhere in East Asia. Editorially, the Tokyo edition of *Nature* will be identical with those printed in Britain and the United States, although the advertisements may differ. For an explanation of the reasons for this development, see page 97. □

UK charities pick up the tab left by government shortfall

London

MEDICAL researchers in Britain are increasingly having to turn to charities to secure funds for projects that the government-financed Medical Research Council (MRC) cannot afford to support. The pressure on the charities, faced with mounting numbers of grant applications, is increasing despite MRC's assertion that "the charities cannot and should not be expected to meet shortfalls in government funding of research". The situation is starkly illustrated with the publication of figures from two of Britain's largest medical research charities, the Wellcome Trust and the Cancer Research Campaign.

Last year's flotation of 25 per cent of Wellcome plc increased substantially the income of the trust, which rose 76 per cent to £57.8 million in 1984-86 compared with the previous two years. Against the background of the shortage of government funds, the publicity surrounding the flotation resulted in what the trust describes in its biennial report "an almost overwhelming" increase in the number of applications for project grants. The trust was forced to expand its system for the assessment of applications and to create new panels of advisers in some areas.

In the past year, the total number of grant applications increased by 50 per cent, although in some subject areas the increase was far greater — a 100 per cent rise is expected this year in biochemistry and cell biology. Approximately half of all applications received meet the required standards for support.

The need for researchers increasingly to seek charitable support is exemplified by Professor David Kirk, of Queen Mary College, University of London. After failing to secure funding from MRC, Kirk successfully applied to Wellcome to support a three-year project to study the effect of inhibitors of ADP-ribosyl transferase on the immune system. "I have had a number of cases in the last two or three years of applications for MRC grants being approved on their scientific merit but not being funded. In that sort of situation I am much more inclined than previously to go to the medical charities for funding."

Wellcome's new-found wealth has now enabled it to direct its efforts to longer-term support of individuals with the creation of research programmes around them. Up to one-third of the trust's income will be used this way, "developing the strength and potential of British medical research". The first manifestation of this new policy was the award of a grant of more than £1 million to the University of Glasgow to set up a unit of molecular

parasitology, guaranteed for five years with the possibility of funding for five more.

At the Cancer Research Campaign (CRC), Britain's leading supporter of cancer research in universities and medical schools, government cutbacks are hampering its own research programme. According to CRC's annual report, despite an increase of 44 per cent in research expenditure since 1984 to more than £25.5 million this year, CRC is experiencing difficulty in meeting requests for funding.

CRC's scientific director, Dr Nigel Kemp, says that over the past two years the number of high-quality grant applications has outstripped CRC's resources to the extent that many approved proposals can no longer be supported. "For the foreseeable future there will be scientific initiatives waiting in the wings." Kemp says the situation has arisen principally because of a reduction in government funding of the universities and MRC, "which means that more campaign money is required to make good deficiencies". From October this year, CRC will increase its stake in the Institute of Cancer Research in London, which it funds jointly with MRC, each providing £3.5 million annually. From 1 October, CRC will take over MRC's share of the funding at an annual rate of 10 per cent, so that by 1997 MRC will no longer contribute.

Although the arrangement was ostensibly agreed to enable CRC to increase its involvement with the institute's research effort, problems were apparent with the joint-funding arrangement, with MRC finding difficulty in maintaining the *status quo*.

Sir James Gowans, secretary of MRC, emphasizes that there has not been a decrease in the number of applications for project grants, and that for the past three years the proportion of successful applications has been increasing, from 38 per cent in 1984-85 to 48 per cent in 1986-87, although many approved applications still do not receive funding. "I am delighted the charities can pick up the good applications we cannot support", he says, while maintaining that MRC by its nature will continue to be the agency that underpins basic medical research in Britain.

The situation is being closely monitored by the Association of Medical Research Charities (AMRC), which recognizes that many of its members are "under a lot of pressure" with increased grant applications. AMRC is particularly wary of suggestions that the government is considering advising universities to charge for the overheads incurred by charity-funded research, a move AMRC sees as directly overstepping traditionally agreed areas of support.

Simon Hadlington

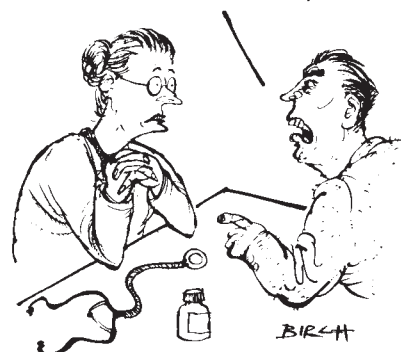
Soviet research "swamped by bureaucracy"

London

SOVIET medical research is being swamped by bureaucracy and over-planning, Health Minister Evgenii Chazov warned recently in an interview on a Moscow radio programme.

Commenting on the need for major "restructuring" in the health sector, Chazov noted that the "scientific plan" for medical research embraces no fewer than 18,000 subjects, under the direction of 72 scientific councils and 509 *ad hoc* committees. The health service has 349 research institutes employing more than 330,000 scientists. Yet the outcome, Chazov said, is "the same old negative trend of extensive growth without qualitative content" that has characterized Soviet life for decades. Even the most eminent establishments are failing to fulfil their promises. For years,

I demand a 295th opinion!



he said, the health service has been calling for the development of Soviet beta-blockers, cephalosporin antibiotics and the like. "But apart from promises, so far nothing has been received."

The main problem, he says, is "the low methodological level of research". "Are there many of our laboratories working today at the cell and molecular level?" "Do many of our institutes have immunology or genetics laboratories? It is these which today largely determine the level of scientific research."

Chazov's recommendations are to abolish bureaucratic bottlenecks, rethink priorities, improve "medical incentives for top-quality personnel" and establish "scientific-production associations". Most important of all, he urged "radical restructuring of the psychology and sense of responsibility of scientific personnel". On this and this alone, he said, the success or otherwise of the restructuring process ultimately depends.

Vera Rich

This is the first issue of *Nature* to be printed in Japan. □

Japan's Human Frontiers project stays in the doldrums

Tokyo

THE future of the Human Frontiers Science Programme, Japan's attempt to forge an international programme of long-term research, seems suddenly to be clouded, in the wake of the last month's economic summit at Venice.

Officials of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry (MITI) have been working for the past two years on this ambitious project, which is aimed at creating new technology through the study of biological functions. Voluminous reports have been produced, and there have been three meetings with scientists of the summit nations, one of them in London on 1 April (*Nature* 326, 8; 1987).

The definition of the intended research programme nevertheless remains stubbornly unclear. Even the staunchest ally of the Human Frontiers project, Prime Minister Yasuhiro Nakasone, criticized the programme as "too vague" when given a draft text to present at the Venice summit meeting. And while the Human Frontiers project achieved a mention at the end of the Venice declaration, participants merely welcomed the initiative, thanked Japan for the opportunities provided for participation in discussions of the programme, noted that the feasibility study was to continue and asked to be kept informed of progress. A commitment to participate in the proposed programme was conspicuous by its absence.

MITI officials say that the Venice summit was "quite successful", although Katsuhiko Umehara, MITI's deputy-director of the programme, admits that the programme is in a "chicken-and-egg situation". If there is no commitment on participation from other summit nations, the Ministry of Finance is unlikely to provide the funds needed for the programme. But other nations are unlikely to join until Japan itself comes forward with a substantial financial commitment to its own programme.

So what is the next step? Umehara says that an "international feasibility study" will be started later in the year, probably in September, with the help of ¥150 million (about \$1 million) of funds already provided in this year's budget. Before then, MITI and the Science and Technology Agency, which is also involved in the Human Frontiers Programme, will be required to submit their budget requests for the succeeding financial year, when they will have to give some plan for the continuation of the project.

Umehara says that MITI and the agency may put forward a "pilot programme" for 1988, incorporating a few typical components of Human Frontiers. Among the projects being considered, he says, is the non-invasive measurement of magnetic fields in the brain using a direct-current SQUID, which would be undertaken by Dr Hisashi Kado's group at MITI's Electrotechnical Laboratory at Tsukuba. Umehara also mentions in this connection the Science and Technology Agency's plan to sequence the human genome (*Nature* 326, 323; 1987).

The Science and Technology Agency, on the other hand, seems to have other views. Masahiro Kawasaki, deputy director-general of the agency's Research and Development Bureau which has charge of the genome project, says that the agency will proceed separately from the Human Frontiers project in 1988 because the human genome project is an urgent matter in which Japan has an agreement on co-ordinated action with the US Department

of Energy, whereas the Human Frontiers project is, at this stage, only a feasibility study. If the Human Frontiers programme is eventually realized, he said, the human genome project could then be incorporated within it.

The essential difficulty in carrying through the planning of the Human Frontiers project seems to be the large numbers of agencies with a finger in the pie. The project was born in the Agency of Industrial Science and Technology in response to the criticism that Japan gets a free ride in technology that springs from basic research in the West.

But the project has grown into a bureaucratic monster involving several ministries and an army of academic advisers inclined to wait and see (which may not be surprising, given that they had not thought of the programme in the first place). One person who could lead the project forward is Prime Minister Nakasone, but he is due to retire in October and none of his potential successors shares his interest in the life sciences.

The fate of the Human Frontiers project should become clear at the end of next month, when the ministries will put forward their budget proposals. David Swinbanks

Sodium leak threatens French nuclear power programme

Paris

A LEAK of liquid sodium from a storage tank at the world's largest fast-breeder reactor, *Superphénix* at Creys-Malville near Lyons, may jeopardize the planned expansion of France's nuclear energy programme. While there seems to be no immediate environmental danger, finding and repairing the leak could take 2-3 years and cost an estimated FF400 million (£40 million). Growing disaffection from nuclear energy among the French public will make the government's handling of the incident a sensitive issue during the run-up to next year's presidential elections.

Meanwhile, the leak will be used as ammunition for those critics who say that the prototype *Superphénix*, designed to produce 1,242 MW of electricity, was a poor investment. It may also stymie the plans of *Electricité de France* (EDF) to build a sister reactor, *Superphénix-2*.

The leak at Creys-Malville was first detected in March this year, when sodium began leaking into the space between the tank and its outer protective shell at the rate of 500 litres a day. Although the leak stopped of its own accord some weeks later, on 25 June it reappeared, at a rate of 800 litres a day. Engineers are now sure there is a 1-mm hole in the base of the tank. Either the sodium will have to be drained from the tank or the whole tank will have to be replaced.

The immediate question to be decided by energy minister Alain Madelin is whether the reactor can continue to function with the tank out of action. Although not part of the steam-raising circuit, the tank is used for storing spent fuel rods removed from the reactor. Experts at EDF argue that, as the new fuel rods being installed during the current maintenance period have a working life of 400 days, they would last long enough for the tank to be drained and repaired. A report being prepared by EDF should allow a decision to be reached at the end of August.

Part of the trouble descending on the French government is a consequence of its handling of the Chernobyl accident last year. At first, the information put out by the official body responsible for radiological safety claimed that the radioactive cloud had avoided France. When it became clear that this was not the case, people began to question the statements on nuclear safety issued by the government. Since then, mounting criticism of French nuclear policy from neighbouring countries has affected public opinion. West German complaints of pollution of the Mosel river by the French Cattenom reactor is causing an outcry, while there has been a decline of the public support for nuclear energy, within France itself, on which the expansionist nuclear programme depends. Peter Coles

Sakharov criticizes Soviet academy

London

ACADEMICIAN Andrei Sakharov last week publicly reproached the Soviet Academy of Sciences for failing to support him during his seven-year exile in Gor'kii. Members of the academy, Sakharov said, had "spread lies" about him and his wife and their well-being. Furthermore, in 1983, three members of the academy had published an article about him which was, he said, an "outright provocation".

Sakharov said that he hoped that the new policies of *glasnost* (openness) and *perestroika* (restructuring) would lead the Academy to "participate in the worldwide struggle of scientists for the liberation of prisoners of conscience"; and criticized restrictions on place of residence and freedom to travel.

Sakharov was speaking at the French Embassy in Moscow, at a ceremony conferring on him honorary membership of the French Academy of Sciences. Sakharov had not been given permission to go to Paris, and he criticized the Academy for refusing to co-organize a Franco-Soviet colloquium to mark the event. Vera Rich

Europeans lose in superconductivity

London

EUROPEAN scientists, anxious not to be left behind in the international race to develop the new high-temperature superconductors, are urging the European Community (EC) to begin a coordinated research effort in both the theory and applications of the new ceramics. This request emerged from a recent meeting in Genoa, organized by the Community, at which progress and prospects for the future were reviewed by 500 scientists representing institutions throughout countries in the EC and the European Free Trade Association (EFTA). As at the Berkeley meeting two weeks ago (see *Nature* 328, 14; 1987), it was clear that despite the man-hours devoted to superconductor research, knowledge of the physical characteristics of the ceramic oxides is in its infancy; present research consists largely of fact-gathering.

The scientific community would like to see specific research programmes aimed at finding new materials, elucidating their properties, and working towards applications in both electronics and electrical engineering. In a move that echoes recent action by the US Department of Energy (see *Nature* 327, 356; 1987), it was also recommended that the Community should set up an information network and database to enable researchers to harness their efforts more effectively. David Lindley

European research programme at heart of budget row

London

THE budget for the five-year European research and development programme, Framework, has once more failed to gain approval from the 12 member states but instead has found itself at the heart of a bitter conflict which has isolated the United Kingdom from the rest of Europe.

Europe's scientific community had hoped that the two-day summit in Brussels last week would sanction the £4,500 million needed to support joint research programmes in the European Economic Community (EEC), designed for projects in information technology, biotechnology, AIDS and cancer, among others.

But even before the leaders formally meet, there were signs that conflict was inevitable and that the science budget



would fall victim to the British decision to veto approval of the total EEC budget for the coming year unless major reforms were forthcoming.

At the heart of the conflict is Prime Minister Margaret Thatcher's view that much of the EEC budget is mis-spent and that too much — now 70 per cent — is used to subsidize the Community's farming, a policy that greatly benefits France.

The political bartering of France and Germany was much in evidence on the evening before the summit, and even at breakfast-time on the first day, when meetings were supervised by Chancellor Helmut Kohl of West Germany and President François Mitterrand of France, in an attempt to find an unofficial solution. While Britain has shown a marked reluctance, since the end of last year, to approve the new science budget — 3,000 research jobs in information technology alone are said to be under threat if early approval is not given — the latest conflict could mean another six months at least before the 12 states have an opportunity to agree.

The conflict and renewed veto of the research and development budget has surprised many in the UK high-technology industries who were convinced that the third administration of Thatcher's government would approve the research and development budget without question. Such confidence seemed to be confirmed by the sacking of the previous information technology minister Geoffrey Pattie — a new team has been installed at the Department of Trade and Industry (DTI) led by Lord Young, a 'political heavyweight' and a confidant of the Prime Minister.

Since last November, the EEC has been threatening to drop the joint research project unless the member states could agree. Nine months later, it has neither dropped the projects nor approved the necessary increased spending. The information technology programme, Esprit, is now six months behind schedule and other major projects are now expected to suffer.

Britain's justification for vetoing the proposed budget is complex. Apart from its dislike of the CAP and the methods adopted for the management and the approval of research programmes, it is at odds with the needs in aid and financial relief of many member states, particularly those from the poor and less industrially developed countries. Thatcher's government, which has been notorious in its opposition to increased public expenditure, is faced with a Community that now seeks to be substantially more than a free marketplace for its members' produce and products. Those ambitions now extend to increasing substantially regional development grants.

On her return from Brussels, Thatcher agreed that spending could continue at its present level — the annual research and development budget is 1,040 million ECU (European Currency Units, 1 = £0.71) — and in the long term her government would seek better control procedures on spending "by seeing they are translated into regulations before we consider any increase in spending".

Bill Johnstone

● Officials at the British National Space Centre (BNSC) have met Lord Young, and are confident that the government will respond positively to their proposals by the end of this month. Unless it receives approval then, claims BNSC, Britain will be unable to take part in the immediate research projects planned by the European Space Agency. These include programmes for the Ariane space rocket and the Hermes space plane. □

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UK Geological Survey offered alternative for a new future

London

A RADICAL change of the relationship between the British Geological Survey (BGS) and its present sponsor, the Natural Environment Research Council (NERC), is advocated by a committee under Sir Clifford Butler whose report* is published this week. The committee says that both the status and the independence of BGS should be enhanced by separation from NERC and by direct accountability to a senior minister, such as the Secretary of State for the Environment.

That BGS has been in trouble has been known for some time (see *Nature* 311, 499; 1984). Its core task of carrying out and publishing a geological survey of the United Kingdom was at one stage nearly squeezed out of existence by the pressure to provide research commissioned by government departments and by industry at a time when its funds and manpower were being reduced. Although the subvention from NERC has recently been increased, the Butler committee considers that these steps will not remedy the situation. Among specific criticisms are the following:

- Publication of BGS survey maps is too infrequent, partly because senior survey staff are perfectionists. BGS has let lapse the arrangements under which groups of experts concentrate on specific areas, becoming in the process over-dependent on generalists.

- NERC has paid too little attention to the maintenance of survey work, while government departments have had too much influence on BGS policy.

- The Butler committee "has the impression" that the NERC council lacks the "time and distinctive expertise, especially of market needs", to provide "helpfully and in detail" an appraisal of BGS's programmes. It acknowledges that NERC has other demands upon it, but says that the secrecy surrounding the findings of invigilatory visting groups "limits the value of their work".

- The appointment of a Director of Earth Sciences at NERC's headquarters is described as "highly disadvantageous" in that it entails "the interposition of another layer of management above BGS and the consequent diminution of the role of its director".

Specifically, the committee says that BGS should be guaranteed £18 million a year (index-linked) exclusively for its programme of structural, geochemical and hydrological surveys, including the development of a digital National Geo-

sciences Data Centre. BGS would also be encouraged to sell data in its control, keeping the receipts, and would be allowed to make contracts with government departments and industrial companies provided the former included a surcharge of 20 per cent (to help with basic research) and that the latter were struck at commercial rates to ensure that BGS could not compete unfairly with private survey companies.

The Butler committee offers alternative schemes for a new organization, preferring that in which BGS would acquire an independent corporate identity somewhat like the present constitution of the British Ordnance Survey. Failing that, the committee would prefer to see BGS attached to a government department than to remain with NERC.

The report was commissioned jointly by NERC and the Advisory Board for the Research Councils, the second of which is likely to be responsible for recommending which course of action should be followed. The committee considers that a change of status could be carried through in time for the financial year beginning in April 1989.

Philip Campbell

* Report of the study group into geological surveying.

French commission

Paris

FOLLOWING the violent reception of his predecessor's attempt to institute reforms in French higher education, Jacques Valade, minister for research and higher education has set up a panel of 69 academic advisers. Divided into five working groups, the panel will prepare a document entitled *Tomorrow's University* for discussion at the end of 1987. Themes for the respective working parties are 'evaluation', 'research and transfer of technology', 'scientific employment', 'the provinces' and 'research funding'. The aim of the commission and its report is to reevaluate, "from scratch", the role of the University in France.

P.R.C.

India off in parallel

Bangalore

INDIA is to begin a new project aimed at the indigenous development of parallel computing systems. The Prime Minister, Rajiv Gandhi, has authorized the Science Advisory Council to launch the project this year, with the goal of producing a 50 megaflop [million floating-point operations per second] machine within three years. A budget of \$14.25 in foreign exchange and R12.85 crore in Indian currency is expected.

R.R.

AIDS workers back on speaking terms

Washington

THE controversy over the potential of peptide T as a treatment for AIDS (acquired immune deficiency syndrome) remained unresolved after a special meeting at the National Institute of Mental Health (NIMH) last week. But the meeting did succeed in returning a conflict dramatized by the media to the realms of normal scientific debate — and opinions did change.

No new data were presented to change substantially the views of the Food and Drug Administration, which will still give permission for clinical trials, provided that certain technical details are provided by NIMH.

Controversy over peptide T erupted at last month's Third International Conference on AIDS when several laboratories claimed that they had been unable to replicate the findings of the discoverer of peptide T, Dr Candace Pert of NIMH (*Nature* 327, 449; 1987). Pert has published evidence that peptide T can inhibit the entry of human immunodeficiency virus (HIV) into cells. In her view, peptide T blocks viral entry because it corresponds to the sequence that binds the HIV envelope glycoprotein (gp120) to the cell surface receptor (T4 or CD4).

That hypothesis is now under siege. Dr Joseph Sodroski and colleagues at the Dana-Faber Institute and Dr Larry Lasky at Genentech analysed the regions of the gp120 envelope glycoprotein responsible for binding. Sodroski reports that by introducing mutations into the envelope glycoprotein he finds three regions are involved, none of them containing peptide T. And in straightforward syncytial assays already published in the *Lancet*, Sodroski shows that peptide T does not interfere with gp120-CD4 binding.

These experiments do not, however, call into question the evidence that peptide T has some antiviral action. Work by Elaine Thomas at Genetic Systems Inc. and by Pert's collaborators confirm that peptide T can inhibit viral infection in a number of different assay systems. To see the effect the experimental conditions have to be exactly right. Thomas's work clearly shows that antiviral effects present at low concentrations of virus (themselves compatible with biological levels) disappear at higher levels. Experimenters who have not found the effect may have been on the wrong part of the curve.

Researchers now seem likely to look for non-specific antiviral effects of peptide T in cells which do not bear the CD4 receptor.

Alun Anderson

Costs prompt West German second thoughts on space programme

Munich

A PREDICTED doubling of the costs for Hermes, Columbus and Ariane — the pillars of Europe's space programme — has created confusion and doubt in the West German government. It is now unclear whether West Germany will continue to support the projects, administered by the Paris-based European Space Agency.

The three projects will cost about DM30,000 million over the next ten years, of which West Germany is to pay almost one-third. A decision on participation is due when the Bundestag reconvenes in the autumn.

West Germany doubled its 1987 budget for the three projects in late June, lending credence to the idea that support would continue at this higher level. But there was speculation during the first week of July that the Bonn government would request a five-year delay in the Hermes space shuttle project. Such a move would seem to favour the development of the two alternative European shuttles, the British *Hotol* and the West German *Sänger*. But this will anger France, the strongest advocates of Hermes.

The claim that West Germany would call for a delay in the Hermes project was denied by an official in the Ministry for Research and Technology (BMFT). Nevertheless, extending the programmes over a longer time-span is one of the most reasonable suggestions for dealing with the massive cost increase. BMFT cannot provide the extra funds, and the West German Finance Ministry is presumed to be reluctant to open its pursestrings.

The challenge to Hermes comes from powerful figures such as Wolf-Michael Catenhusen, chairman of the Research and Technology Committee of the Bundestag. Catenhusen disapproves of Hermes because it is "just a small version of the US space shuttle" which will be long outdated by the time it is put into service in 1995. By this logic, a delay in Hermes could well mean its end.

Catenhusen, a Social Democrat (SPD), makes more general charges, complaining that the ruling conservative coalition has approved expensive space projects without having a comprehensive policy. BMFT's proposal on 24 June to establish a West German space agency seems a tacit acknowledgement that the government has indeed been weak in this regard.

But SPD science spokesman Josef Vosen continues to support German participation in Hermes as well as Columbus, the European section of the manned space station to be built by the United States. Vosen fears the political consequences of pulling out of either Hermes or

Columbus. He accused the supporters of *Sänger* as an alternative to Hermes of "living in a dream world".

Vosen supported his arguments by citing the low level of support for the space programme in West Germany: \$14 per inhabitant a year compared with \$22 in France and \$100 in the United States. Previous cooperative projects such as Spacelab, financed by West Germany, used for two missions, and then given to

the United States, are proof, he says, that Europe needs an independent space programme. Spacelab was a case, Vosen said, where "We [and the US] ate the cake together, but we paid for it ourselves."

West German participation in the European space programme is ultimately a question of political will. Arguments that West Germans spend too little on space flight seem unlikely to succeed with the electorate. One more failure of the Ariane rocket could create insurmountable opposition to any new initiatives, leaving politicians in the difficult position of choosing whether to please their allies or their constituents.

Steven Dickman

Lakes on the way to recovery from long-term acidification abuse

London

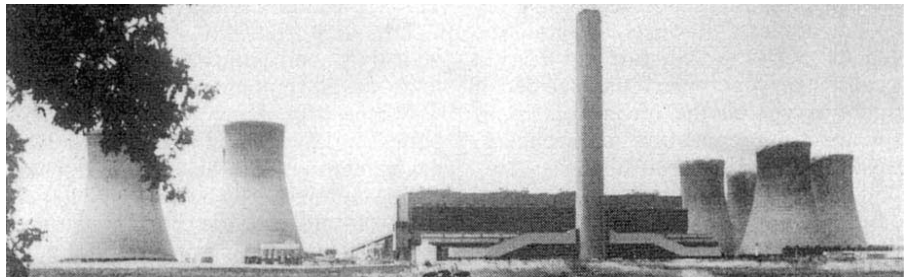
BRITISH and Scandinavian scientists have discovered the first indications of recovery in some lakes acidified by sulphur emissions.

Scientists working in the Surface Water Acidification Programme of the Royal Society of London, the Royal Swedish Academy of Sciences and the Norwegian Academy of Science and Letters have just published the results of their first three years of work, after a conference last week in Bergen. Studies of diatoms in lake

gramme. "Planting forests has changed the acidity of waters in all three countries we studied", Mason says.

Forests contribute to acidification by the filtering action of their needles, which capture mist and fog particles; by taking up magnesium and calcium in their roots and replacing it with hydrogen; and by altering drainage patterns when planted in previously non-forested areas.

What the scientists call "short sharp episodes" can have a lethal effect on fish populations; when acidity is increased



The 650-foot multiflue chimney with cooling towers at the CEGB's Fiddler's Ferry power station.

sediments revealed progressive acidification of lakes in Norway, Sweden and Scotland from 1850 until about 1970, with a slight decrease since 1975 when sulphur emissions from Britain began to decline.

"There is no doubt that we have to reduce sulphur emissions to reduce acidification", said the director of the programme, Sir John Mason of the University of London's Imperial College. "We still have some questions to answer on how long it will take to show any effect, and that depends on the soil type and other factors."

The research shows a strong correlation between areas of high acidic deposition, acidified surface waters and the decline of fish populations. Measurements of pH, aluminium and calcium in surface water will give an indication of whether fish will live or die, the scientists have concluded.

The role of forests in water acidification has also been examined by the pro-

gramme. "Planting forests has changed the acidity of waters in all three countries we studied", Mason says.

Britain's Central Electricity Generating Board, which is funding the five-year programme, says the results so far are consistent with earlier information which led it to a decision to install flue gas desulphurization (FGD) at three of its 40 power stations. The first FGD will be retrofitted at the Drax power station in Yorkshire by 1993, with planning work still going on for the other two units.

Mason says that "the fact that the scientists say we must remove the sulphur will underpin the CEGB policy."

Kathy Johnston

This is the first issue of *Nature* to be printed in Japan. □

Aborigines halt Woomera teams' supernova observations

Sydney

A GROUP of desert-dwelling aborigines has derailed US and West German plans to use rocket-borne X-ray and ultraviolet observations of the supernova in the Large Magellanic Cloud. Both teams had planned to use the Woomera rocket range, but half of the 260-km target zone overlaps with land belonging to the Maralinga Tjarutja aborigines. The aborigines have now refused permission for anybody to enter to recover spent rockets or scientific instruments.

The Maralinga Tjarutja people are resolutely denying access despite assurances from the Ministry of Defence in Canberra that members of the tribe would be allowed to accompany recovery parties to ensure the sanctity of their sacred sites. They are also unmoved by the information that their settlement is well outside the target area and by the assurances of the

science minister, Mr Barry Jones, that nobody has ever been injured by any of the more than 800 rockets launched from Woomera since the early 1950s.

The aborigines have bitter memories of past experiments. They were forcibly evicted from their lands for British nuclear tests between 1956 and 1963. They were allowed back only three years ago, when the South Australia state parliament added to their title to the land the right to govern access to it.

According to the Maralinga Tjarutja solicitor, Mr Darcy O'Shea, the plan for Woomera seems like "a rerun of the 'it's only desert' mentality of the 1950s". He says that the aborigines consider it incompatible with their ownership of the land that part of it should also be part of a rocket range.

Negotiations between the Ministry of Defence, on behalf of the Ministry of

Science, and the Maralinga Tjarutja people are continuing. It may be possible for the rocket observations to be carried out by using less than ideal trajectories which avoid the tribal lands.

Supernova 1987A has given Woomera a new lease of life. The fortunes of the facility reached a low point two years ago, when more than 130 buildings, including the missile-test workshop, were sold off. Activity at Woomera was at a peak in the mid-1960s, but the range was last used in March 1979. Much of the facility has been in mothballs ever since.

Although formal contracts have not yet been signed, the British Skylark rocket that West German scientists plan to use for X-ray observations of the supernova is already on a ship. Launch is planned for mid-August. West Germany is considering a five-year programme of observations.

The United States, represented by the National Aeronautics and Space Administration, plans a series of eleven launches beginning in mid-October, with a possibility that the programme would continue for ten years. US balloon-borne observations of gamma-rays have already begun from Australia's launching station at Alice Springs.

Charles Morgan

UK advanced information technology further delayed

London

A DECISION on the future of Britain's national programme of research and development into advanced information technology is to be further delayed because the government has decided it cannot accept all the recommendations contained in a report it commissioned to consider a successor to the five-year, £350-million Alvey programme.

But a commitment has been given for a further national programme. The government will now set up another committee, called IT '92, to consider the findings of the report on a post-Alvey programme, produced by a committee led by Sir Austin Bide. The Bide report was published in November, and a decision on its recommendations had been expected soon after last month's general election. Last week, Mr John Butcher, under secretary of state at the Department of Trade and Industry (DTI), said the government did not accept the Bide report "lock, stock and barrel".

The report had recommended a £1,000-million collaborative programme, with the government providing £425 million for a five-year effort focusing largely on the applications of information technology in user industries. About £300 million of the government contribution would be put into the research effort, with 50 per cent funding for industrial partners and 100 per cent for academic research, as with the

Alvey programme. The total research funding would amount to £550 million. The applications scheme would receive £125 million from the government, with industry's contribution bringing the total to about £500 million.

A review of Alvey achievements was published last week in advance of a two-day exhibition later this month, at which some 90 projects will be displayed to persuade customers of the programme's success. The Alvey programme was started in 1983 to develop precompetitive collaborative research in the enabling technologies in response to increased overseas efforts on information technology research, particularly in Japan. The Ministry of Defence, DTI and the Science and Engineering Research Council together provided £200 million, with industry's contribution at £150 million. More than 200 industrial projects have been approved, typically consisting of two or three companies with one or two academic institutions.

Butcher says the success of the programme has confounded its early critics: despite the 'precompetitive' nature, many of the projects had led to products or the prospect of products, and investment in exploitation of the research was four times greater than the original investment.

Alvey's director, Brian Oakley, says that the role of academic researchers in the new programme is likely to be "very different" and that many scientists involved in Alvey's basic research will return to their long-term work. Simon Hadlington

No amnesty for Chernobyl operators

London

THE Chernobyl operators responsible for the disaster will not benefit under the forthcoming amnesty to mark the seventieth anniversary of the October Revolution, according to Evgenii Smolentsev, deputy chairman of the Soviet Supreme Court. Interviewed by the official Soviet news agency TASS some three weeks before the trial of the Chernobyl operators was due to open, Smolentsev explained that the amnesty will not apply to people guilty of "particularly dangerous crimes against the state", such as espionage, smuggling, treason and banditry. This category, he noted, also covers the neglect of safety procedures where there is danger of an explosion, a classification which clearly covers the Chernobyl catastrophe.

The neglect of safety procedures at Chernobyl was, of course, established almost a year ago by the special Soviet government commission of inquiry, and in that respect the main concern of the trial is to apportion the degree of guilt among the accused. Nevertheless, at least one of the operators directly responsible for the explosion is known to have paid for his folly with his life, and it is not inconceivable that the court's verdict might have left the dead to carry the burden of blame. The timing of Smolentsev's remarks, which seem to take the guilt of the living accused for granted, seems curious.

Vera Rich

Doctors vote to test for AIDS without consent

London

DAUNTING legal hurdles stand in the way of doctors who put into practice the new British Medical Association (BMA) policy on testing for AIDS (acquired immune deficiency syndrome) without the patient's consent.

After a stormy debate, the BMA's annual meeting last week voted to support the concept that "testing for HIV [human immunodeficiency virus] antibody should be at the discretion of the patient's doctor, and should not necessarily require the consent of the patient".

The decision, taken on a 180 to 143 vote of the 600 doctors at the meeting, is widely seen as showing more concern for the protection of doctors than of patients. It contradicts the policy of Britain's Department of Health, the World Health Organization and the BMA council.

After the meeting, the BMA said it would advise doctors that ethical considerations should not permit indiscriminate testing and that the "discretion of the doctor" in the new policy must be in the best interests of the patient, not the doctor.

Doctors will also be warned that they could be committing an assault if they take patients' blood to test for AIDS antibodies without consent. The BMA says doctors could be sued, and may face disciplinary action from the General Medical Council, if they carry out AIDS testing in secret.

Doctors at the conference were clearly concerned about their own risk of infection. "I for one feel my life and those of my medical and nursing colleagues are more important than the future insurance and employment prospects of infected individuals", an anaesthetist is quoted as saying. Another doctor invoked the catchphrase of Department of Health publicity on AIDS: "Don't die of ignorance. Test the lot, before surgery at least."

The conference was told that the number of infections in health care workers could increase substantially. A spokesman said afterwards that the BMA has always warned doctors to take precautions against the AIDS and hepatitis-B viruses.

Some AIDS specialists feel the new policy could help to drive underground those most at risk. Because some doctors may begin taking blood samples for HIV testing, patients are being advised to tell their doctor if they do not want the test.

The BMA's decision came under attack from the Medical Practitioners' Union, the Royal College of Nursing and the National Union of Public Employees, which represents 250,000 health workers.

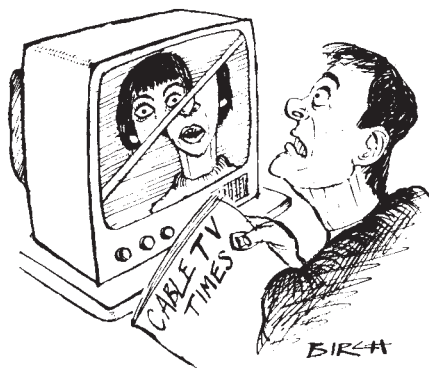
Kathy Johnston

Japanese scientists to begin dialling for seismic signals?

Tokyo

INTERNATIONAL telecommunications companies planning to lay trans-Pacific optical fibre cables may have an unexpected group of customers — Japan's seismologists. The global seismology subcommittee of the Japanese National Committee of Seismology and Physics of the Earth's Interior has proposed that digital earthquake detectors should be permanently installed in submarine telephone cables to be laid in the Pacific in the 1990s. This proposal is part of an even more ambitious plan to establish a network of seismometers covering the Western Pacific from the Aleutian Islands to Indonesia and Papua New Guinea.

The subcommittee was set up in April last year to plan Japan's contribution to



the global seismic network being developed under the aegis of the Federation of Digital Broadband Seismographic Networks. The federation is sanctioned by the International Union of Geodesy and Geophysics to coordinate efforts to install global networks of broadband digital stations such as the French GEOSCOPE project begun in 1981 and the US IRIS project begun last year.

Japan already has several networks of permanent seismometers maintained by government agencies including universities, the geological survey, the geographical survey institute, the National Research Center for Disaster Prevention and the Japan Meteorological Agency. More than 70 sets of data from these networks in the Tokai-Kanto region around Tokyo are telemetered to the headquarters of the Japan Meteorological Agency in Tokyo, and monitored around the clock for earthquake prediction and disaster prevention purposes, but most of the seismometers respond only to ground motions with periods of between 1 and 10,000 seconds, and only a few are under the sea.

The subcommittee proposes that the new network, called POSEIDON (for Pacific Orient Seismic Digital Observation Network) should be established in

two phases. In the early 1990s, land-based seismometers would be installed in Japan, the Asian continent, the islands of the Western Pacific and the East/South-East Asia region, together with a central data centre. At a later stage, a deep-ocean network would be put in place.

The total cost of POSEIDON is estimated at about ¥1,000 million (\$7 million). This year's feasibility study, during which the subcommittee will sound out the opinions of seismologists in the countries covered by the proposed network, is supported with a small grant of ¥3 million from the Ministry of Education, Science and Culture.

A central feature of the POSEIDON plan are broadband seismometers capable of detecting motions with periods ranging from a tenth of a second to a day, including microseisms and Earth-tides. Kunihiro Shimazaki, vice-chairman of the subcommittee, says that a network of such seismometers could "revolutionize" seismology in the western Pacific by providing high-resolution data on processes such as plate subduction, hot-spot vulcanism and flow in the mantle as well as processes occurring during large earthquakes.

But the most novel aspect of the plan is the proposal that seismometers should be installed in submarine optical-fibre cables. Kokusai Denshin Denwa (KDD) and AT&T are vying with Britain's Cable and Wireless to lay trans-Pacific cables in the 1990s (see *Nature* 327, 91; 1987). What the subcommittee suggests is that seismometers should be installed in the pressure housing of the cable repeater stations spaced at intervals of about 100 km along the cables. The repeaters would provide a ready-made power source, while signal stacking and other signal processing techniques would allow the signal-to-noise ratio of the seismic measurements to be considerably enhanced.

How likely is it that these plans will succeed? One subcommittee report says that preliminary discussions with the research staff of one of the international telecommunications companies have been "very encouraging". But it seems that these preliminary discussions have drawn attention to major practical problems that will have to be overcome. One difficulty is that the proposed cables will be jointly owned by several companies in different countries, each of which will have to be separately convinced that ocean-bottom seismometers will not interfere with the operation of their cable.

David Swinbanks & Harry Glicken

This is the first issue of *Nature* to be printed in Japan. □

University of Edinburgh's woes

SIR—Your leading article "Academic wall writing" (*Nature* 326, 114; 1987) rightly points out that universities which regularly receive slightly less than average funding emerge, after a few years, very much worse off than the (itself dismal) average. You conclude that some universities are "on the way down" and may eventually cease to have any role in research, while others may manage to stay afloat.

Your article implies, however, that low funding necessarily means poor research. This is not so. The major factor in determining shifts in the allocation of resources between universities has been what may broadly be termed 'teaching costs'; this has been clearly stated by the University Grants Committee (UGC)¹. The adoption of a 'common funding base' by the UGC means that institutions are now allocated a fixed sum for each student in a given subject, with no variation, such as existed previously, between institutions^{1,2}. This is expected to account for 61.2 per cent of total resources allocated in 1989–90³. Thus, universities that have had above-average costs in the past are penalized while those with lower costs enjoy a relative gain. Many factors contribute to these costs. It is sometimes assumed that high costs betoken idleness, incompetence or other vices, but small classes and old, expensive buildings are more probable interpretations.

The University of Edinburgh is a case in point. You pick it out as having done poorly in the financial distributions. This is true, although several other universities have unfortunately fared still worse, but it is not associated with a low research rating. In the UGC's assessment exercise, some 70 per cent of Edinburgh's departments emerged in cost centres deemed to be above-average for research in the United Kingdom and 10 per cent were rated outstanding. Edinburgh was the fourth largest recipient of external research funds in the United Kingdom in 1984–85, the most recent period for which comparative figures are available⁴, and was in the top 30 per cent even when the figures are corrected to take account of size-differences between the universities. In its letter to the university detailing the basis of the grant for 1986–87, which applies in essence up to 1989–90⁵, the UGC stated specifically that research had told in its favour; the main factors that worked against it were the change to a common funding base and some change in the relative numbers of students taking

different subjects⁶. In addition, the UGC's planned student numbers for Edinburgh in 1989–90 are (for unknown reasons) nearly 3 per cent below the 1984–85 level, against a 1 per cent overall increase for British universities; this means a further relative reduction in funding. Ironically, the three universities that obtained the lowest external research funding per member of staff in 1984–85 (less than one-third that of Edinburgh) have all been treated more favourably than Edinburgh in the latest UGC distribution.

Similar considerations doubtless apply to some other universities that have fared badly under the UGC's new criteria for resource allocation. Not all have been condemned on their research record. Unfortunately all of them will, as you remark, suffer alike in being unable to recruit young people with new ideas.

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1. Letter from UGC to universities, 20 May 1986, para. 15–16.
2. *Review of the University Grants Committee* (Report of a committee under the chairmanship of Lord Croham) para. 3.42 (HMSO, London, 1987).
3. *Review of the University Grants Committee* para. 3.43 (HMSO, London, 1987).
4. *University Statistics* Vol. 3 (University Statistical Record, London, 1984/5).
5. Letter from UGC to universities, 10 February 1987.
6. Letter from UGC to University of Edinburgh, 27 May 1986, Part 2, para. 1.

Primitive myth

SIR—I agree with Bob Johannes¹ that there is a unique knowledge of marine life among Pacific Islanders and that many traditional maritime activities may have helped to conserve marine resources. We should try to understand these customs better and we should be concerned where they are disappearing.

It is also true, however, that since the 1960s there has been something of a revival of the Rousseauesque spirit, a guilt about the way 'Western' manners have spread and affected others, and a desire to see something nobler than our own values in non-Western cultures. We should be careful, however, when looking for evidence with which to support such human rights issues that we do not rely solely on speculation.

In his own book on marine lore of the Palau District of Micronesia in the western Pacific, Bob Johannes (ref.2, footnote p.65) concedes that traditional marine tenure may have come into existence in order to reduce conflict, and not overtly to conserve resources. It is evident from other accounts that such tenure was not necessarily based on rational decisions on fisheries management, and may some-

times have had nothing to do with such tenure at all³.

It is important that we understand the origins of tenure: traditional tenure systems cannot be expected to perform functions in the modern context for which they were not designed. Johannes dismisses evidence for bad resource management⁴, but it is apparent from the response of tenure to new exploitation patterns³ that this traditional marine territoriality has limited adaptability to the modern setting. The question still remains as to why, if many Pacific Islanders were purposefully careful of their shallow-marine biological resources, they were not apparently more often mindful of the even more limited and visible terrestrial ones.

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1. Johannes, R.E. *Nature* 325, 478 (1987).
2. Johannes, R.E. *Words of the Lagoon* (University of California Press, 1981).
3. Polunin, N.V.C. *Senri ethnol. Stud.* 17, 267 (1984).
4. Diamond, J.M. *Nature* 324, 19 (1986).

Problems of Indian science

SIR—Four decades have passed since India won independence from British rule, which was known for its fair administration. Since then, scientists in medical and non-medical disciplines have suffered frustration, indignation and injustice at the hands of their superiors, so much so that a few have even committed suicide. Paradoxically, no Indian prime minister has been able to curb the activities of those responsible for containing the aspirations of young and promising scientists. If even a few had followed the example of the late Dr Homi J. Bhabha, then head of the Indian Atomic Establishment, their suffering would have been minimal.

In the light of claims and counter-claims made by Indian scientists, both at home and abroad, during the past decade, it is clear that all is not well with the scientific community in the country. The formation of the Society for Scientific Values (see *Nature* 326, 535; 1987) reveals the truth that it has been infested with several maladies.

The founder of the society is Dr Avtar Singh Paintal, the new director-general of the Indian Council of Medical Research (ICMR). To my knowledge, he is the first director-general to have taken such a lead. One can only hope that the reputation of the present Prime Minister, Mr Rajiv Gandhi who is popularly known as "Mr Clean", will also help scientists to achieve the goal they have set before them.

M.R. DAREKAR

Swiss National Collection
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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only. □

A celebration of connectionism

New developments in neural network theory have excited both psychologists and neurobiologists. Practitioners of the new art displayed their wares last week.

Oxford

WHEN David Rumelhart, Geoffrey Hinton and Ronald Williams described for neural networks a powerful new learning procedure called back-propagation (*Nature* 323, 533; 1986), they noted that theirs was not a plausible model of how brains learn. Yet the generality of their approach, and the several intriguing features of network learning by back-propagation which have come to light, have stimulated a resurgence of interest in neural network models among neuroscientists, theoreticians and experimentalists alike. Last week, at a meeting organized by the Society of Experimental Psychology, a packed audience heard Geoffrey Hinton describe a new learning algorithm that seems a better model of biological learning than is back-propagation by parallel networks which nevertheless seems to retain much of the power of its predecessor.

In parallel distributed processing, a network can be thought of as embodying a mathematical function mapping vectors in 'input space' to vectors in 'output space', much as matrices effect linear transformations between vector spaces. A vector in the neural context is simply the pattern of excitation of some set of units taken to be the input or the output of the network. The processing is thus distributed in the pattern of the connections between units of the network and their strengths. Corresponding to the real physiological task of, say, pattern recognition, will be some kind of network function mapping inputs (patterns) onto outputs (interpretations). The all-important question is what kind of network is needed for a particular task.

Simple networks developed in the 1960s, known as perceptrons, involved only two layers of units, an input and an output, with direct connections between them. Such networks are very limited in the range of tasks they can carry out. The versatility of a network can be greatly increased by the introduction of intermediate layers of 'hidden' units, but this raises the problem of how it can be trained.

Back-propagation provides an elegant way of training a multi-layered network. During learning, the output vectors generated by the network for a given input are compared with the desired output, giving an error calculated from the difference between the two. Back-propagation calculates the dependence of this error on all the connection weights, simply by using

the chain-rule for differentiation, and the weights are adjusted to reduce the error, so that the network converges by gradient descent on the required structure.

During learning, the network comes to capture certain general features which are characteristic of its task. The hidden units, in particular, develop features that seem especially significant to neuroscientists who record the properties of single neurons in brains. For example, in some cases they are reminiscent of the way in which some neurons in the brain are found to be specific for different aspects of the representation of the visual field.

Even so, this system of learning by back-propagation has not seemed very biologically realistic. Hinton (Carnegie-Mellon University) and his colleagues have been looking for a more plausible system of learning.

The new development is known as a 'recirculation' algorithm, and works as follows. In a network learning by back-propagation, there is a linear flow of activity (via the hidden intermediate units) from the input units to the output units. In the new system, the hidden units connect back to the single layer of 'visible' input units. Activity thus recirculates through the network; during training, the connection weights are adjusted to minimize the rate of change of activity in each unit. Thus, when trained, the network is set up so as to stabilize on certain states, and so can work as a kind of 'content addressable memory' with the property that degraded or incomplete forms of the training inputs can regenerate the correct version.

It has been shown that, under certain conditions, the new algorithm is equivalent to gradient descent, and it has been found empirically that the system still works when these conditions are relaxed.

A number of interesting applications of back-propagation were reported at the meeting. Hinton described a network for recognizing one-dimensional shapes on a one-dimensional retina independently of position: the hidden units learn to respond to shapes in different positions. Hinton also described a speech-recognition network which learns to recognize spoken consonants given very noisy corrupted data. It appears to perform almost as well as people, and better than the previous best system of automated speech recognition.

Several speakers described analogies between the behaviour of their networks during training or after 'damage' and what

is known of human learning and cognitive disorders. For example, J. L. McClelland (Carnegie-Mellon University) described how a network for learning a balance-beam task progressed through stages of competence similar to those of children given the same task. The problem is to decide which way a balance-beam will tip, depending on the position and size of weights on either side of the fulcrum. With an appropriately biased learning environment, such as children might well experience, the network, like children, initially bases its decisions purely on weight information; gradually the network learns to use the position of the weights. M.S. Seidenberg (McGill University) described a network for word recognition and pronunciation that, when 'damaged' by the removal of hidden units, displayed behaviour reminiscent of some human disorders, such as dyslexia.

Parallel distributed processing is not without its critics, and S. Pinker (MIT) reported that a linguistic analysis of Rumelhart and McClelland's network for changing the tense of verbs in sentences shows that the system is not 'descriptively adequate' as a model for human language, in that it abandons certain symbolic rules and principles that linguistic studies suggest are crucial in human language.

David Willshaw (Edinburgh) asked whether parallel distributed processing networks might, like perceptrons, similarly cease to make significant progress and fade in interest after a period of development and excitement. McClelland's riposte was that work on perceptrons was severely limited by the available computer power and circumstances are now sufficiently different to justify optimism.

The biological relevance of parallel distributed processing remains an open question. Independently of relevance, however, work on network systems may be of interest at a purely theoretical level. The present work is a kind of experimental mathematics, and in that respect is rather similar to that of Mandelbrot on fractals, also made possible and inspired by computers. The hope is that, in future, deductive proofs will give a more rigorous basis to work on networks.

Many interesting problems remain. On what set of functions will a given network topology converge? How can the optimal network for a given task be predicted, and how long will training take? And so on.

Geoffrey North

DNA structure

Physicists retreat again

Maxim Frank-Kamenetskii

SINCE the discovery of the DNA double helix by J. Watson and F. Crick in 1953 this beautiful structure has attracted a great deal of attention, not only because of its fundamental role in biology. Time and again, physicists have approached DNA with insufficient caution, hoping to fit it into their scheme of the world. Is it a semiconductor? Or even, perhaps, a superconductor, and at room temperature at that? Or has it anomalous magnetic properties? There has hardly been a novelty or a vogue among solid-state physicists that has not been applied to DNA. But the helix-coil transition (DNA 'melting') is, perhaps, the only successful attempt worth mentioning. By contrast, the list of failures is impressive. The data reported by Gabriel *et al.* on page 145 of this issue¹ add one more to this sad list.

For physicists, the double helix has always resembled a solid. They never forget Schrödinger's prophetic words² "We believe a gene — or perhaps the whole chromosome fibre — to be an aperiodic solid". DNA does look like a solid. The base pairs are arranged as in a one-dimensional crystal, in which each base pair is flanked only by two neighbours. The DNA crystal is aperiodic because the sequence of base pairs is as irregular as the sequence of letters in coherent printed text. And like letters in text, the AT and GC base pairs are similar in both width and height.

No wonder, then, that physicists are so intrigued by the one-dimensional DNA crystal, a crystal entirely unfamiliar, and have often bravely attempted to take DNA over from the biologists and to subject it to a purely physical treatment.

In their most recent attempt to hijack DNA, physicists have used a new sophisticated weapon, the soliton, which is the current vogue in many branches of physics. Solitons, or solitary waves, are solutions of some nonlinear wave equations. They differ from ordinary waves (which are solutions of linear equations) by their unusual stability.

Solitons in DNA were first invoked to explain the unexpectedly long lifetimes (up to one second) of the open state of DNA found using the hydrogen-exchange method.³ Recently, however, Guéron *et al.*⁴ have shown that these long lifetimes resulted from a misinterpretation of the hydrogen-exchange data. Revised estimates lead to quite reasonable lifetimes in the microsecond timescale, as I described⁵ in a News and Views article last week.

The next claim was connected with the observation by Edwards *et al.*⁶ of sharp

resonances in the microwave-absorption spectrum of DNA solutions. The effect was so striking that the enthusiasm of the theorists is understandable (see the News and Views article⁷ by John Maddox). Particularly impressive was the fact that the peak intensities depended dramatically on DNA supercoiling. For soliton enthusiasts, the data left no doubt as to the reality of solitons in DNA⁸. At the same time, Van Zandt claimed that the data should be treated within the framework of linear theory, that is, without solitons^{9,10}.

While theorists were hotly debating which model, linear or nonlinear, better

fitted the data of Edwards *et al.*, two groups of experimentalists embarked on a painstaking verification of the data. In their joint article in this issue¹ they report that they find no difference in microwave absorption between DNA solution and a pure buffer without DNA: the hijackers have failed again. □

1. Gabriel, C. *et al.* *Nature* **328**, 145–146 (1987).
2. Schrödinger, E. *What is Life?* (C.U.P., 1944).
3. Englander, S. W. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 7222–7226 (1980).
4. Guéron, M., Kochoyan, M. & Leroy, J.-L. *Nature* **328**, 89–92 (1987).
5. Frank-Kamenetskii, M. *Nature* **328**, 17–18 (1987).
6. Edwards, G. S., Davis, C. C., Saffer, J. D. & Swicord, M. L. *Phys. Rev. Lett.* **53**, 1284–1287 (1984).
7. Maddox, J. *Nature* **324**, 11 (1986).
8. Scott, A. C. & Jensen, J. H. *Phys. Lett. A* **109**, 243–245 (1985).
9. Van Zandt, L. L. *Phys. Rev. Lett.* **57**, 2085–2087 (1986).
10. Van Zandt, L. L. & Davis, M. E. *J. biomol. Struct. Dyn.* **3**, 1045–1053 (1986).

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Genetic engineering

Bacteria conjugate with plants

Conrad Lichtenstein

AGROBACTERIUM tumefaciens, a soil bacterium, is a natural genetic engineer of plants. It contains a large tumour-inducing (Ti) plasmid, a portion of which (T-DNA) is transferred to the nuclear genome of infected plant cells. These transformed cells express T-DNA-encoded genes which results in cell proliferation producing plant tumours called crown gall disease (see ref. 1). Last year a paper published in *Nature* provided strong evidence that the mechanism of T-DNA transfer is similar to bacterial plasmid conjugation². Now, on page 172 of this issue³, Buchanan-Wollaston *et al.* provide convincing evidence for a common mechanism for these two phenomena by showing that the mobilization functions of a naturally occurring bacterial plasmid required for plasmid conjugation also allow transfer of this plasmid to plants by *A. tumefaciens*.

In plasmid conjugation, as exemplified by the F factor (see ref. 4), one strand of a double-stranded plasmid molecule is unidirectionally transferred from donor to recipient bacterium. This requires cell-cell contact brought about by gene products encoded by plasmid transfer (*tra*) genes. During transfer, one strand of the plasmid is nicked by a plasmid-encoded site-specific endonuclease within a *cis*-essential transfer origin, *oriT*. The nicked strand is unwound in a 5'-to-3' direction by a helicase activity and transferred as a DNA-protein complex to the recipient. DNA synthesis produces a replacement strand in the donor and a complementary strand in the recipient. Smaller non-conjugative plasmids can exploit the

transfer machinery of conjugative plasmids for transfer to a recipient, but require their own *oriT* and mobilization (*mob*) proteins.

In the Ti plasmid, the T-DNA is flanked by two 25 base-pair direct border repeats, which define the T-DNA element for transfer. The right border (RB) alone can signal T-DNA transfer but the orientation of this sequence is critical as it specifies the direction of transfer. Transfer requires recognition of susceptible (wounded) plant cells; specific phenolic compounds produced by such cells⁵ trigger the transcriptional activation of the Ti-plasmid virulence (*vir*) genes whose products effect T-DNA transfer⁶. Induction of *vir* results in the generation in *A. tumefaciens* of a site-specific endonuclease cleavage (by *virD*-encoded gene products) on a common strand at each of the border repeats^{5,7–9}. Then a free strand-specific copy of the T-DNA, the T-strand, is produced in these cells⁹. This molecule probably exists in the bacterium as a protein-DNA complex and may be the intermediate that *A. tumefaciens* transfers to target plant cells.

A comparison of the two systems suggests functional analogies between: (1) *oriT* and the T-DNA border repeats; (2) the mobility (*mob*) genes and the *virD* genes; and (3) *tra* and *vir* genes required for cell-cell recognition, production of the DNA-protein transfer complex and production of the structure through which the complex is transferred.

In their paper³, Buchanan-Wollaston *et al.* extend these functional analogies by showing that the *oriT* and corresponding

mob genes of the small non-conjugative plasmid, RSF1010, can substitute for the requirement for a T-DNA border for transfer to plant cells. They used *A. tumefaciens* strains containing a helper plasmid to supply *vir* functions *in trans*, and one or other of a variety of small plasmids constructed *in vitro* and encoding a kanamycin-resistance gene capable of expression in plants. Plant tissue infected by such strains grows on media containing kanamycin if the resistance gene is transferred to the plant genome.

These experiments showed: (1) plasmids containing both *oriT* and *mob* will transfer the drug marker to plant tissue with similar frequencies to plasmids containing the T-DNA borders; (2) the *oriT* sequence alone is neither sufficient for DNA transfer to plants nor for conjugal transfer of the plasmid to a recipient bacterium; and (3) transfer in both cases is restored by supplying *mob* functions *in trans* from a third plasmid. Also, *in vitro* transcription and translation experiments show that three proteins are encoded in the RSF1010 *mob* region, and an *in vitro*-generated frameshift mutation in the largest coding region abolishes DNA transfer to plants.

These results are important for the construction of the next generation of Ti-plasmid-based vectors for plant genetic engineering. Ironically, it was previously supposed that the inclusion of T-DNA borders was essential. Clearly, this requirement is now obviated. These results also raise some interesting biological questions. Do plasmids with *oriT* and *mob* of RSF1010 still require the *virD*-encoded endonuclease? One prediction is that they do not, unless this endonuclease has additional functions specific for T-strand synthesis and/or transfer. Can a T-DNA border plus *virD* replace the requirement for *mob* and *oriT* in plasmid conjugation? How general is the phenomenon they have revealed: are other plasmids similarly transferred by *A. tumefaciens* to plants? If so, there are profound implications for the role bacteria might have played in plant evolution. The agro-bacterial/plant interaction has always been presumed to be unique; however, might other prokaryotes have the ability to transfer conjugally specific DNA elements to particular target eukaryotes? □

1. Lichtenstein, C. & Fuller, S.L. in *Genetic Engineering* Vol. 6 (ed. Rigby, P.W.J.) Ch.3 (Academic, London, 1987).
2. Stachel, S.F., Timmerman, B. & Zambryski, P. *Nature* **322**, 706–712 (1986).
3. Buchanan-Wollaston, V., Passiatore, J.E. & Cannon, F. *Nature* **328**, 172–175 (1987).
4. Ippen-Ihler, K.A. & Minkley, E.G. *A. Rev. Genet.* **20**, 593–624 (1986).
5. Stachel, S.F., Messens, E., Van Montagu, M. & Zambryski, P.C. *Nature* **318**, 624–629 (1985).
6. Stachel, S.E. & Nester, E.W. *EMBO J.* **5**, 1445–1454 (1986).
7. Yanofsky, M.F. *et al.* *Cell* **47**, 471–477 (1986).
8. Wang, K., *et al.* *Science* **235**, 587–591 (1987).
9. Stachel, S.E., Timmerman, B. & Zambryski, P.C. *EMBO J.* **6**, 857–863 (1987).

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Mass extinctions

How sudden is sudden?

Stephen K. Donovan

THE essential feature of a mass extinction, as seen in the fossil record, is that many organisms or groups of organisms die out during a period that is geologically instantaneous, even though it may represent many years in real terms. Our understanding, however, of such sudden geological events is often confused by the use of a coarse geological timescale, where all the organisms that disappeared during a particular era are lumped together, whether they died before, during or after the mass extinction. Detailed interpretation of the fossil record of certain groups of extinct molluscs shows that a large extinction event about 65 million years ago, that at the Cretaceous–Tertiary (K/T) boundary, attributed to the effects of a single bolide (meteor) impact, was preceded by a pattern of smaller disappearances.

A particularly complete K/T boundary sequence is exposed in a superb coastal outcrop at Zumaya, north Spain. Detailed studies of bivalves and ammonites (an extinct group of cephalopods) from this section reveal an unexpected pattern of extinction¹. The novelty of this approach is that most K/T boundary sections are defined on the basis of microfossils and/or dinosaurs.

At Zumaya, oyster-like inoceramids bivalves, whose fossils are large and distinctive even when fragmentary, died out during the last 8 million years of the Cretaceous era, the Maastrichtian. The orthodox view now is that this group became extinct at the K/T boundary, yet its demise seems to predate this event by several million years. Perhaps stranger still is the pattern of ammonite distribution at the top of the Cretaceous section. There are three hitherto unrecognized ammonite assemblages at the top of the lower Maastrichtian and in the upper Maastrichtian but the youngest ammonites disappear about 10 m below the K/T boundary.

Indeed, no K/T sequence is known where the ammonites survive to the mass-extinction horizon¹. The inoceramids at least disappear at a recognized boundary, defined by the demise or evolutionary change of other organisms. The end of the ammonites cannot be correlated with any other biotic event. Although the gradual decline of the ammonites in the Upper Cretaceous has long been recognized², it was previously believed that their final demise was a result of the mass extinction.

Another group of molluscs that show an abnormal pattern of extinction during the Maastrichtian are the benthonic rudists, a group of reef-building bivalves that some-

times reached gigantic proportions. For example, the Maastrichtian species *Titanosarcolites giganteus* reached a maximum length of 2 m, and *Durania nicholasi* was the size of a large waste-paper basket. The rudists were limited to shallow, tropical seas and reached their greatest diversity during the Maastrichtian^{3–5}. Yet they were extinct by the beginning of the Tertiary, and many rudist groups perished before the K/T boundary. In Jamaica, for example, there is very good evidence that the last rudists were killed by volcanic, rather than extra-terrestrial, events before the end of the Cretaceous.

It has been suggested⁶ that the rudists were largely extinct one or two million years before the K/T mass extinction and that their latest Maastrichtian faunas were small and isolated. If so, then they may have been particularly susceptible to changes in the environment, which could have led to their demise at the end of the Cretaceous. The most important trait for the survival of a large taxonomic group at the time of a mass extinction appears to have been a broad geographical range, with members of the group inhabiting numerous basins⁷. Thus the late Maastrichtian extinction (for which the mechanism is not known) greatly reduced the diversity and range of the rudists, so that the weakened stock was not strong enough to survive the effects of the K/T bolide impact.

If we look at the K/T impact as an event that destroyed certain already weakened stocks, we still do not have an explanation for the process of the earlier Maastrichtian extinctions. The late Cretaceous–early Tertiary, however, was a time of global tectonic activity, with events such as the opening of the Atlantic affecting global ocean-water circulation and both creating and destroying habitats. Perhaps this was the true coffin-maker of mass extinction, with the K/T bolide driving the final nail home for many groups □

1. Ward, P., Wiedmann, J. & Mount, J.F. *Geology* **14**, 899–903 (1986).
2. Kennedy, W.J. in *Patterns of Evolution, as Illustrated by the Fossil Record* (ed. Hallam, A.) 251–304 (Elsevier, Amsterdam, 1977).
3. Masse, J.-P. & Philip, J. *Bull. Centres Rech. Explor.-Prod. Elf-Aquitaine* **10**, 437–456 (1986).
4. Jones, D.S. & Nicol, N.J. *Paleont.* **60**, 107–115 (1986).
5. Nicol, D. *Nautilus* **100**, 69–71 (1986).
6. Kauffman, E.G. in *Cretaceous–Tertiary Boundary Events II* (eds Christensen, W.K. & Birkelund, T.) 29–37 (University of Copenhagen, 1979).
7. Jablonski, D. *Abstr. 3rd Int. Congr. Syst. Evolut. Biol.*, **84** (1985).

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Intelligence testing

Bryter still and bryter?

Chris Brand

PSYCHOLOGISTS are having to pay a heavy price these days for preferring the abstractions of 'cognition' to the mundanities of IQ. In France, a nuclear physicist has turned his hand to demonstrating that social class of rearing has an effect — albeit a modest one — on child IQ (*Nature* 325, 767; 1987). Now, a political scientist at the University of Otago, New Zealand, Professor James Flynn, has brought to fulfilment a most scrupulous programme of international scholarship through the mails by demonstrating "massive" increases in raw scores on intelligence tests in 14 economically advanced nations over recent decades (*Psychological Bulletin* 101, 171–191; 1987).

On culture-fair tests of fluid intelligence, Flynn's remarkable data — deriving especially from the testing of hundreds of thousands of military conscripts in western Europe — show gains in scores equivalent to 18 IQ points (one standard deviation) per generation in countries and locales where trustworthy and comparable data are available as far back as 1950. The table provides an illustration: for Dutch draftees, mean IQs increased by 20 points over a 30-year period. Altogether, Flynn's work substantiates the observation of "accelerative secular development of psychological characteristics" that was first made by Liv Karen Koppen-Thulesius and Helfried Teichman (*Br. J. soc. clin. Psychol.* 11, 284–294; 1972), and that was first documented in the case of Japan by Richard Lynn (*Nature* 297, 222–223; 1982).

Flynn draws two main conclusions. First, he observes that massive IQ-type gains are possible without psychologists having the foggiest idea as to their cause: he estimates that only 3 of the 20 IQ points gained in Holland can be attributed to rising socio-economic levels, for example, so what Americans now call 'poor demographics' were not our ancestors' primary problem. Second, Flynn urges that IQ is a trivial variable in human terms, for these large IQ gains have not been accompanied conspicuously by recorded improvements in educational attainments or other intellectual advance: from 1960 to 1980 the number of patents granted in Holland fell by a third. By implication, the IQs of western minority groups (notably those of Afro-Americans) might easily be increased, but without doing much real good; and present ethnic differences on the tests should invite fresh suspicion that they are cultural in origin.

Whether the secular changes have been anything more than 'what the tests test' now requires urgent consideration. On

the usual psychometric assumptions, such changes in mean scores should have had quite enormous, visible effects at the tails of the normal distribution of IQ. As Arthur Jensen remarks (in Modgil, S. & Modgil, C. *Arthur Jensen: Consensus and Controversy* 379–381; Falmer, Brighton, 1987), the Dutch IQ gain implies that there would have been approximately 11 times as many retarded persons (with IQs below 70) in the Netherlands in 1952 as there were in 1982. Similarly, Dutch university entrants (assuming they are the top 10 per cent of the population) should now have an average IQ of 140 by the standards of 1952.

Yet it is precisely Flynn's point that, because IQ might be trivial (measuring mere problem-solving ability), Dutch professors cannot be expected to be found rejoicing in the streets. It scarcely matters,

Intelligence levels (on a 40-item version of Raven's Progressive Matrices) among all tested* 18-year-old draftees in the Netherlands at points over 30 years.

Year	Per cent passing more than 24 items	Mean IQ (using 1952 as baseline)
1952	31	100
1962	46	106
1972	63	112
1981/2	82	121

*According to Flynn, "...the percentage who fail the medical exam and escape testing has remained constant [at about 20%] from 1952 to 1981/2 and should not be a significant source of error".

according to Flynn, that IQ scores may have increased thanks to test-sophistication gamesmanship, superior skills and sensitivities on the part of testers, tester laxity, improved nutrition, increased outbreeding, increased assortative mating by people of higher IQ, urbanization, health care, 'teaching the tests', increased opportunities for young people in advanced countries to select and create their own appropriate experiences and environments, declining family sizes, declining use of lead plumbing, or whatever. The fact is that IQ itself just does not seem to matter very much.

For better or worse, this second conclusion of Flynn's cannot possibly survive the wider series of empirical tests to which it can be subjected. J.E. and R.F. Hunter (*Psychol. Bull.* 96, 72–98; 1984), for example, have reported IQ to be the major predictor of occupational success in the United States, despite occupational psychologists having laboured for decades to stress the importance of other factors in the testing of which they could have found gainful, more congenial employment.

So why, then, do IQ scores increase across generations? One answer may be

found in the increasing permissiveness, liberalism and extraversion of the 'advanced' economics which may have given their progeny a special boost on culture-fair IQ tests. Such tests are often given under time limits that hardly encourage reflection; and, of course, they were not designed to credit the assiduous application, accuracy, attention to detail, organization and feats of memory that might once have favoured the educated classes. IQ tests were designed to penetrate such artificialities: so it is perhaps not surprising if they now record gains as education takes a less meticulous form in which speed and intelligent guessing receive encouragement in the classroom. Comparison of secular trends on different types of intelligence tests would allow evaluation of this possibility.

Yet what if the IQ rise is entirely genuine? And what if Flynn's sober view of modern educational achievements can be substantiated in times when students must, after all, be admitted to micro-compute, hang-glide, speak psychobabble and smash guitars aesthetically as never before? Even this would strictly say nothing about within-generation differences — including group differences. By way of example, infants suffering hypothyroidism are readily boosted in IQ by medical endeavour; yet perfectly real IQ differences are well preserved among such children, after the treatment (Fishler, K., Galiker, B.V. & Koch, R. *Am. J. Mental Defic.* 69, 515–525; 1965). Flynn's 'unknown' intergenerational change, factor X, like an increased educational stress on quick, intuitive, rough, uncritical, poorly articulated and mis-spelled answers to questions, may have dashed the larger aspirations of testers towards culture-free (and generation-free) measurement of intelligence without making the tests any the less appropriate for comparing one victim of educational liberalism with another. Flynn has located what could be the Achilles heel of mental testing; but it remains to be seen whether the final identification of factor X will give this heel more than a scratch. □

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100 Years ago

ON the matter of dispersion equivalents, Dr J.H. Gladstone holds that the following conclusions are warranted by the accumulated data: (1) That dispersion, like refraction, is primarily a question of the atomic constitution of the body: the general rule being that the dispersion equivalent of a compound is the sum of the dispersion equivalents of its constituents. (2) That dispersion, like refraction, is modified by profound differences of constitution, such as changes of atomicity. (3) That the dispersion frequency reveals differences of constituents at present unrecognized by chemists, and not expressed by our formulae.

From *Nature* 36, 239; 7 July 1887.

Cosmology

Radio-dating the Galaxy

Gerry Gilmore

THE determination of the age of the Universe is one of the most important, but most difficult, problems in modern astronomy. In standard models, the age of the Universe depends primarily on the present rate of expansion (measured as the Hubble constant) and deceleration rate, which is determined by the total mass in the Universe. Thus, comparison of the Hubble age of the Universe with an independent age is a test of cosmological models. The most commonly used alternative measure of age is derived from the oldest stars known, using stellar evolutionary theory, giving, of course, only a lower limit to the age of the Universe. On page 127 of this issue, Harvey Butcher provides a new calibration of the stellar evolutionary age scale, by comparison with radioactive dating of stars near the Sun, that suggests a large systematic error in the current models. If correct, both the accepted physics of stellar evolution and the age of the Universe require substantial revision.

The technique of age dating using radioactive elements was devised by Rutherford (*Nature* 123, 313–314; 1929), and is now a well-known technique. The principle is straightforward: radioactive parent atoms decay into one or more daughter elements at a rate controlled by a measurable constant, the half life. Thus if one counts the relative number of parent and daughter elements, and knows the half life, it is easy to deduce the time at which the pure parent element was created. In practice, for astrophysical dating it is also necessary to know the initial (relative) abundance of parent and daughter elements. As heavy elements are continually created from lighter ones (by stellar nuclear burning) throughout the lifetime of the Galaxy, it is also necessary, if comparing old and new stars, to allow for the rate of creation. Because of these problems, as well as the observational difficulty in measuring spectral features from rare elements, considerable uncertainties remained in previous analyses, and the derived age of the Universe fell in the range 10–25 gigayears (Gyr).

The most useful radioactive pair for cosmology so far has been $^{232}\text{Th}/^{238}\text{U}$, as the half life for ^{232}Th is 20 Gyr, similar to the time of interest. Rather than measure the ratio of these elements, Butcher has compared the elemental abundance of ^{232}Th directly with that of a stable element, neodymium (mostly ^{142}Nd), which is relevant as its spectral line of interest is excited in the same part of the stellar atmosphere as that of thorium. Thus,

effects specific to the atmospheres of individual stars are minimized.

The price paid, however, is that thorium and neodymium are created in different astrophysical processes, so that the time dependence of their relative abundance becomes problematic. Thorium is created in the r-process, involving the rapid absorption of neutrons in a very high neutron-density environment. This process is poorly understood, but probably occurs mostly in rapid helium burning in supernovae and novae. Neodymium is formed mostly by s-process, involving the slow absorption of neutrons in a lower neutron-density environment. This occurs mostly in the normal late evolution of intermediate-mass stars. A second time-dependent source of thorium is, of course,

PLANS to launch space sculptures into Earth orbit to celebrate the centenary of the Eiffel Tower have caused great alarm among astronomers — see for example the recent News and Views article of Paul Murdin (*Nature* 326, 125; 1987). But even now, the purely functional satellites that populate the heavens are interfering with observations, as highlighted by a recent letter in *The Astrophysical Journal* (317, L39–L44; 1987) from Paul D. Maley of the Rockwell Shuttle Operations Company. The illustration, which comes from that paper, emphasizes dramatically how crowded space has become. It shows a computer-generated 'snapshot' of all the catalogued satellites at 00.00 hours Universal Time on 1 January 1987.

In particular, Maley considers flashes of light recorded photographically and also observed by eye in the vicinity of the constellations Perseus and Aries (MacRobert, A. *Sky Telesc.* 70, 54; 1985; Katz, B. *et al. Astrophys. J.* 317, L33–L37; 1986). It was considered that these flashes, lasting about 1 second, might be caused by flare stars, or γ -ray or X-ray bursters. For this reason there was interest in the magnitude (intensity), spectra and light curves (time variation of intensity) of the flashes. But Katz *et al.* showed that the flashes were too frequent to be caused by such sources or by meteors.

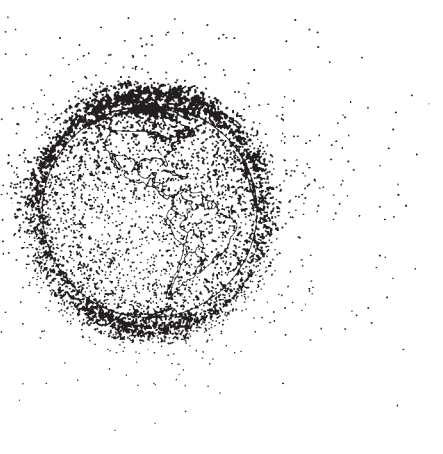
In his analysis, Maley investigated the probability that the events were caused by reflections of the sun off satellites. For example, for one event on 19 March 1985, he traced the trajectories of 5500 objects,

the decay of its parent nuclei. Each of these sources will occur with a natural timescale which is the age of our Galaxy, the Milky Way, and so needs careful consideration when using a sample of stars in the Milky Way disk, as Butcher has done.

At face value, Butcher's excellent data show no decay at all in the Th/Nd relative abundance. This implies a short age scale, with the oldest stars being no more than 12 Gyr, rather than 18 Gyr, old. The absence of any change in the ratio is surprising, and suggests that careful consideration of the relative production histories of r- and s-process elements is necessary. If the conclusion is correct, however, the implications are profound. Not only is the currently accepted physics of stellar evolution incomplete, but the Universe may be substantially younger than presently believed. Extension of this experiment to elements with different production histories and half lives is eagerly awaited. □

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of which some 1400 were above the local horizon within the hour at which the observation was made. There are two possible candidates to be the source of the flashes, the most likely being Cosmos 1400. Telescopic observation of specular reflections of sunlight of Cosmos 1400 confirmed Maley's suspicion. Maley could similarly account for three other flashes that he chose to investigate. A further three flashes, from a separate observation



(Pedersen, H. *et al. Nature* 312, 46–48; 1984) remain unexplained and could originate from the supernova remnant N49, as originally postulated.

There is burgeoning interest in studies of short-duration phenomena such as flare stars, but the investigations are in their infancy, requiring large-angle, high-resolution detectors to identify these infrequent events. As the skies become more cluttered, it seems that astronomers' patience will be increasingly tested — what price space sculptures? □

G proteins

Control of exocytosis

Robert D. Burgoyne

THE generation of intracellular signals in response to occupation of membrane receptors involves, in many cases, a GTP-binding protein (G protein) that is an essential component of the transducing machinery controlling an enzymatic activity or ion channel. Thus, adenylate cyclase is controlled by stimulatory (G_s) and inhibitory (G_i) G proteins; cyclic GMP-phosphodiesterase is activated by transducin in photoreceptor cells; breakdown of polyphosphoinositides by phospholipase C is controlled by the putative G protein G_p ; and certain membrane receptors control potassium or calcium channels directly through an as yet unidentified G protein. At a recent meeting* evidence was presented suggesting that exocytotic secretion from different cell types is controlled by yet another G protein.

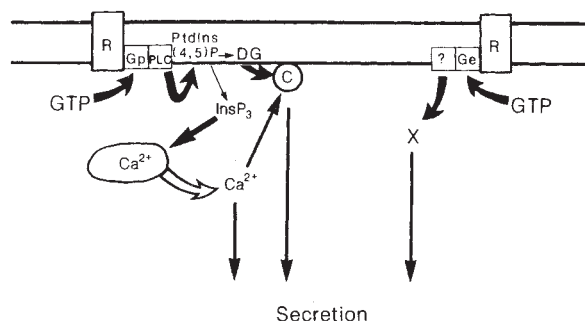
The importance of G proteins was recognized from the requirement for GTP in transduction processes and by the ability of non-hydrolysable analogues of GTP such as $GTP\gamma S$ or $GppNHp$ to turn on these systems irreversibly. Work on permeabilized cells shows that GTP analogues regulate secretion but the manner in which this control is expressed varies from cell to cell. This variation could result from the involvement of multiple G proteins, a question that is yet to be fully resolved. One obvious mechanism for the action of GTP analogues in secretory cells is activation of phospholipase C through G_p leading to generation of diacylglycerol and inositol trisphosphate ($InsP_3$) (see figure). Release of calcium from intracellular stores by $InsP_3$ is unlikely to be of consequence in permeabilized cells as $InsP_3$ would be diluted by leakage from the cells and intracellular calcium is usually well buffered in such experiments. Activation of protein kinase C by diacylglycerol could be significant because in many secretory cells activation of protein kinase C enhances secretion by reducing the requirement for calcium. It is now clear that in some cell types GTP analogues regulate secretion both through G_p and protein kinase C and through a separate G protein whose action is not mimicked by phorbol esters.

In neutrophils (B. Gomperts, University College London) GTP analogues stimulate secretion even in the absence (less than $10^{-10}M$) of calcium and do so even if phospholipase C activation is prevented by pretreatment of the cells

with pertussis toxin before permeabilization. Calcium-stimulated secretion could also occur through a G protein as it is inhibited by $GDP\beta S$, a competitive inhibitor of G-protein activation. Intriguingly, in these cells only one of the two classes of secretory granules exocytoses in the absence of calcium in response to GTP analogues, thus allowing the possibility of selective control of secretion from each class of granules.

Calcium-independent stimulation of secretion by GTP analogues has also been demonstrated in permeabilized cells of the rat insulinoma cell line RINm5F (C. Wollheim, University of Geneva). This

Possible mechanisms by which GTP analogues could regulate exocytotic secretion in permeabilized cells. The first possible site of GTP action is the G protein (G_p) that controls phospholipase C (PLC). Activation of phospholipase C would result in the breakdown of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P$) to inositol trisphosphate ($InsP_3$), which would release calcium from intracellular stores, and diacylglycerol (DG) which would activate protein kinase C (C) leading to secretion. A second site of action of GTP analogues has been established and the G protein involved termed G_c . The most likely role of G_c is in signal transduction from receptors (R), generating a novel second messenger (X) that controls secretion.



effect can be dissociated from activation of protein kinase C as secretion still occurred at low calcium levels ($10^{-10}M$) despite the fact that $InsP_3$ production could no longer be detected. In addition, the GTP analogue $GppNHp$, which effectively elicits secretion, stimulates little $InsP_3$ production.

An effect of GTP analogues very similar to that in neutrophils and RINm5F cells is also observed in permeabilized cultured bovine adrenal chromaffin cells (R. Holz, University of Michigan), suggesting that a similar G-protein mechanism occurs in all three cell types. However, in freshly isolated bovine chromaffin cells, GTP analogues inhibit calcium-dependent secretion (D. Knight, King's College London) and, therefore, in this cell type maintenance in culture could modify the intracellular control mechanisms.

In mast cells GTP analogues give varying results depending on the method used for introducing them into the cell interior. In mast cells accessed using the patch-clamp technique in the whole-cell mode (R. Penner, Max-Planck Institut für Biophysikalische Chemie, Göttingen) GTP is required for calcium release from

intracellular stores in response to the secretagogue compound 48/80. $GTP\gamma S$, however, can elicit degranulation directly in cells in which calcium is strongly buffered by calcium chelators, indicating the presence of a calcium-independent pathway for the action of $GTP\gamma S$. Calcium alone is insufficient to trigger exocytosis as $InsP_3$ injection elicits a transient calcium response but no secretion. These results demonstrate an obligatory requirement of activation of a novel G protein for secretion. The situation in mast cells permeabilized by treatment with streptolysin O is somewhat different. Secretion from these cells again demonstrates an obligatory requirement for activation of a G protein, but GTP analogues elicit secretion only in the presence of elevated calcium levels (Gomperts). Again, this response to GTP analogues can be dissociated from protein kinase C action as it occurs in the absence of any

nucleotide substrate for the kinase and when phospholipase C is inhibited by neomycin.

A possible identification of a *ras* proto-oncogene product as the G protein controlling exocytosis came from further studies on mast cells (D. Bar-Sagi, Cold Spring Harbor Laboratory). A polypeptide of relative molecular mass 21,000 is the endogenous *ras* proto-oncogene product in mast cells, and microinjection of a human *ras* oncogene product results in massive degranulation of mast cells. The mechanism of action of the *ras* protein is not known, but in fibroblasts microinjection of this protein stimulates phospholipase A_2 activity¹. It is intriguing that botulinum toxin type D, which inhibits exocytosis in adrenal chromaffin cells², has recently been shown to ADP-ribosylate a polypeptide of relative molecular mass 21,000 (ref. 3). Is the G protein in exocytosis, the *ras* proto-oncogene product and the botulinum-toxin substrate one and the same protein? The connection may be fortuitous but this speculation is certain to be tested soon.

The mystery G protein involved in the control of exocytosis was tentatively

*Exocytosis: Post-Receptor Events in Secretory Cells 5th Cell Surface Research Fund Meeting, London 9–10 April 1987.

named G_i by Gomperts, implying that it is directly involved in membrane fusion in exocytosis. It is known, however, that in one secretory cell, the sea urchin egg, GTP analogues have no effect on exocytosis in isolated cortical membrane preparations (M. Whittaker, University College London) and therefore a G protein is unlikely to be involved in exocytosis in this system. As we have suggested in a recent News and Views article⁴, GTP analogues could regulate secretion through an effect on the cytoskeleton. By analogy with the known G proteins I think it most likely that the new G protein functions in signal transduction leading to the production of an as yet unidentified second messenger (see figure) that controls exocytosis and perhaps other cellular functions.

It is appropriate that much of the excitement at the meeting stemmed from findings made using permeabilized cells. Peter Baker of King's College London,

who was to have spoken at the meeting, made many significant findings in his work on calcium regulatory mechanisms. In recent years he was one of the pioneers of the use of permeabilized cells to gain access to the cell interior and his laboratory made several major contributions to the study of exocytotic mechanisms. Following his untimely death on 10 March 1987, the meeting was dedicated to his memory. The study of secretory mechanisms, and indeed physiology in general, will not be the same without Peter Baker's energetic and individual contributions. □

1. Bar-Sagi, D. & Feramisco, J.R. *Science* **233**, 1061–1066 (1986).
2. Knight, D.E., Tonge, D.A. & Baker, P.F. *Nature* **317**, 719–721 (1985).
3. Ohashi, Y. & Narumiya, S.J. *Biol. Chem.* **262**, 1430–1433 (1987).
4. Burgoyne, R.D. & Cheek, T.R. *Nature* **326**, 448 (1987).

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Neuron membranes

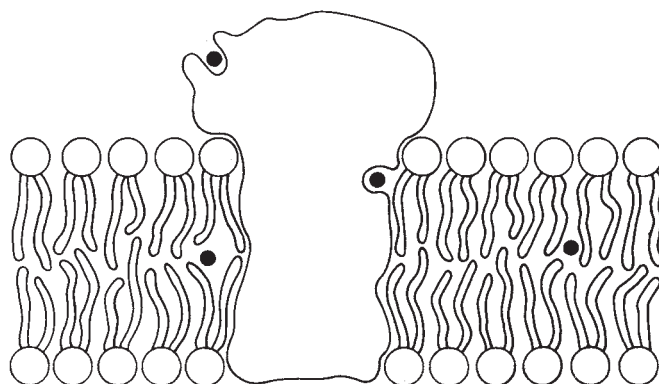
Anaesthetics on the mind

N.P. Franks and W.R. Lieb

ANYONE who has had the misfortune to undergo general anaesthesia knows that it can be only a matter of seconds between the introduction of the anaesthetic and oblivion. What will not be known by the patient, or indeed by anyone else, is how general anaesthetics exert their effects or even the nature of their target sites in the brain. On page 157 of this issue¹, Evers, Berkowitz and d'Avignon report the results of experiments designed to give direct information on the molecular environments of anaesthetic molecules in the brain during general anaesthesia. They reach the surprising conclusion that, during the rapid induction of anaesthesia in rats with the widely used inhalational agent halothane, almost all the anaesthetic molecules are highly immobilized by binding to saturable sites in the brain. They consider their results as evidence against the popular notion that anaesthetics act by nonspecific perturbation of nerve membranes (see figure).

At every level of the mystery of how and where general anaesthetics act, major uncertainties exist. Even at the gross anatomical level, no particular regions of the brain have been identified as the primary targets. At the cellular level, although there

is a fairly wide consensus that anaesthetics act at synapses rather than along the nerve axons, the evidence for this view is certainly not overwhelming. At the subcellular level, both circumstantial evidence and historical precedence have led to the belief that anaesthetics act



There is no general agreement as to the molecular nature of general anaesthetic target sites in the brain. The traditional view has been that general anaesthetics (black circles) act by nonspecific perturbation of the lipid portions of nerve membranes. An alternative view is that these small molecules bind to hydrophobic pockets on proteins (which possibly, but not necessarily, are membrane-bound). In between these two extremes, anaesthetics may act at interfacial sites between proteins and lipids. In their paper in this issue¹, Evers *et al.* present new evidence on the molecular environment of anaesthetic molecules in the brain which may provide an important clue as to the molecular nature of general anaesthetic target sites.

directly on membranes and, moreover, on the lipid regions of these membranes². But there is now a substantial body of evidence^{3,4} supporting the view that anaesthetics in fact act directly on proteins.

The experiments of Evers *et al.*¹ bear directly on the subcellular nature of general anaesthetic target sites. Using ¹⁹F nuclear magnetic resonance, they monitored the uptake of halothane (CF₃CHClBr) into the brains of rats undergoing general anaesthesia. This method⁵ has the major attraction of being able to monitor halothane levels not only in excised brains but also in the living animal. After appropriate (although rather involved) calibrations, Evers *et al.* estimated the average brain concentrations of halothane in rats breathing halothane vapour at concentrations up to 4 per cent (v/v). In addition, they obtained information on the molecular environments of the halothane molecules by measuring the spin-spin relaxation times T_2 of the ¹⁹F atoms. (T_2 is a sensitive indicator of molecular motion.) From their measurements, they conclude that most of the halothane molecules in the brain were highly immobilized and bound to saturable sites that were half-occupied at the same inspired halothane concentration that induces anaesthesia.

What do these new results mean in terms of the molecular nature of the anaesthetic-binding sites? Most workers would have anticipated, and there is some evidence⁶ to support this view, that most halothane in the brain would be found dissolved in the lipid portions of membranes. But Evers *et al.*, although identifying a lipid-like environment (on the basis of their T_2 values), show that only a small fraction of the halothane is found there after the rapid induction of anaesthesia.

Could the halothane in the highly immobilized sites also be in a lipid environment? Although possible, the observation of saturability seems to exclude this suggestion. (We know of no lipid system that shows saturation of halothane at these concentrations.) Nonetheless, the nature and properties of lipids at protein/lipid interfaces are poorly understood, and it is just conceivable that saturable lipid sites exist in these regions.

Alternatively, could most of the halothane in the brain be binding to protein molecules? This would certainly account naturally for both the relative immobilization and the observation of saturation, but is it quantitatively reasonable? Evers *et al.* estimate that the maximum binding

capacity of the highly immobilized sites is about 1.2 mmol halothane per litre of brain. Taking the protein content of rat brain to be about 10 per cent (w/w), it follows that, on average, there would have

to be one site for each protein molecule (of average relative molecular mass 100,000) in the brain.

Whether or not this is plausible is difficult to judge, as so few data are available on the binding of halothane to proteins. Although there are certainly examples⁷ of proteins that are inhibited by halothane and have binding sites that saturate at low anaesthetic levels, they have been thought to be few and far between. It may well turn out that many more proteins can bind halothane and other anaesthetic molecules at surgical concentrations without this affecting their normal functions. This may prove rather difficult to test directly, however, as aqueous concentrations of halothane during surgical anaesthesia are so high (several hundred micromolar). To measure binding to protein sites which half-saturate at these concentrations using conventional direct binding methods, it would be necessary to use similarly high concentrations of proteins. For most pure proteins, this is rarely practical.

Nonetheless, there is little doubt that the observation of abundant saturable binding sites in the brain is quite unexpected and runs contrary to most current thinking. How secure, then, is the demonstration of saturation by this new technique? Even in small rodents, equilibration between the blood and brain can take hours⁸, and changes in cerebral blood flow could therefore give rise to apparent saturation. In dogs⁹ at least, halothane increases cerebral blood flow at inspired concentrations between 0.5 and 2 per cent, but between 2 and 4 per cent (in the region where saturation is observed¹) there is a marked decrease in blood flow back to control values.

It appears possible, therefore, that the reported saturation is caused by physiological rather than chemical factors. No doubt in the near future several groups will be addressing these problems by measuring halothane uptake in thin brain slices or brain tissue homogenates¹⁰, where the problems of blood/brain distribution and non-equilibrium can be circumvented. If the results of Evers *et al.* are then confirmed, they will be seen to represent an important step towards unravelling the mystery of general anaesthesia. □

1. Evers, A.S., Berkowitz, B.A. & d'Avignon, D.A. *Nature* **328**, 157–160 (1987).
2. Miller, K.W. *Int. Rev. Neurobiol.* **27**, 1–61 (1985).
3. Richards, C.D. in *Topical Reviews in Anaesthesia* Vol. 1 (eds Norman, J. & Whitwam, J.G.) 1–84 (Wright, Bristol, 1980).
4. Franks, N.P. & Lieb, W.R. *Nature* **300**, 487–493 (1982).
5. Wyriticz, A.M. *et al. Science* **222**, 428–430 (1983).
6. Bárány, M., Chang, Y.-C. & Arús, C. *Biochemistry* **24**, 7911–7917 (1985).
7. Franks, N.P. & Lieb, W.R. *Nature* **310**, 599–601 (1984).
8. Duncan, W.A.M. & Raventos, J. *Br. J. Anaesth.* **31**, 302–315 (1959).
9. McDowall, D.G. *Br. J. Anaesth.* **39**, 186–196 (1967).
10. Coburn, C.M. & Eger, E.I. *Anesth. Analg.* **65**, 960–962 (1986).

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Developmental neurobiology

Uncertainties in the retina

J.H. Scholes

THE number of different kinds of neurons in the vertebrate brain can only be guessed at, but is certainly very large. How is this vast cell diversity unfolded during development? On page 131 of this issue¹, Turner and Cepko describe the important new step they have taken to begin unravelling cell lineages and determination in the development of the central nervous system, starting with its beautifully formed sensory outpost, the retina (Fig. 1).

The authors used a murine retrovirus vector to integrate and express the *Escherichia coli* β -galactosidase gene in mammalian cells, for use as a lineage marker². Scattered clones of retinal cells were infected in the rat eye at birth, then retrieved and identified by staining histochemically for the bacterial gene product once retinal development was complete. The marker, apparently unnoticed by the cells that harbour it, replicates with the host genome at mitosis, is cell autonomous, and can be expressed in a wide variety of neural and somatic cells.

retina turn to making other cell types during retinal regeneration after damage⁴. By contrast, cell fate is rigidly determined during mosaic development in early invertebrate embryos, and the mechanisms involved could presumably operate elsewhere and in conjunction with regulative ones. Some form of genomic clock could in principle register successive mitoses and programme gene expression during mosaic-type cleavages, but random chromosome segregation⁵ must preclude accurate operation of mechanisms of this sort. This limitation could be what places the onus for fate determination in mosaic development upon cytoplasmic parcelations and daughter-cell interactions.

Neuronal cell lineages have been studied mainly in invertebrate brains, which contain precise assemblies of cells identifiable from one individual to the next. In the most notable case, the nematode worm *Caenorhabditis elegans*, cell type is given by mitotic pedigree in the exact lineage trees mapped in this animal⁶.

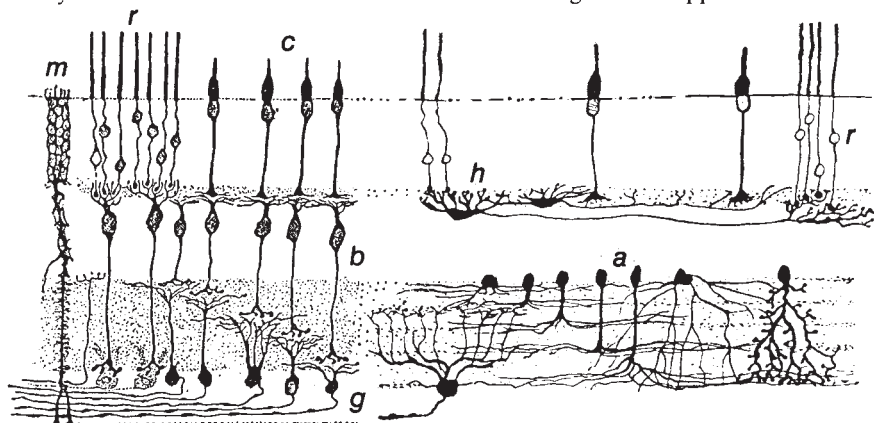


Fig. 1 Ramon y Cajal's two impressionistic schemata¹¹ of the cellular organization of the vertebrate retina combined with slight changes, to show the variety of bipolar (b), amacrine (a), and ganglion (g) cell types; also the rods (r), cones (c), Müller glia (m) and horizontal cells (h).

The clones analysed are small ones, formed by rare integration events late in retinal histogenesis, near its completion. Despite this, they contain an unexpectedly wide range of cell types, in rather random combinations. These striking results are interpreted as meaning that mitotic lineage *per se* may play no part in detail in determining the identities of the retinal cells, which must therefore read their fates from the surrounding tissue.

Various external regulative mechanisms determine cell fate during development, and an example in the nervous system is that cultured glial precursor cells can be induced down either of their two alternative astrocytic differentiation programmes by choice of culture medium³. Closer to home, rod precursor cells in fish

Even here, however, determination is not the rule, and there is good reason for caution in evaluating deterministic appearances. In the most precise of all invertebrate cell assemblies studied, the *Drosophila* eye, the watershed observations of Lawrence and Green⁷ showed that small clones of lineage-related cells in this near-crystalline structure have random composition, indicating that the various retinal cells must read their identities interactively, not from their mitotic *curriculum vitae*. How this could work is suggested by recent findings^{8,9} on the cell-homoeotic mutant *sev*, where the seventh and last photoreceptor (R7) recruited to each developing facet fails to differentiate as such. The *sev* target gene product is transiently expressed in the developing

cell clusters of the facets and its sequence has membrane-receptor properties. It could be the wild-type molecule that R7 must read during the intricate up-and-down dance of the photoreceptor precursors in the eye disk.

Turner and Cepko favour the view that similar principles operate in rat retinal histogenesis, explaining the wide and

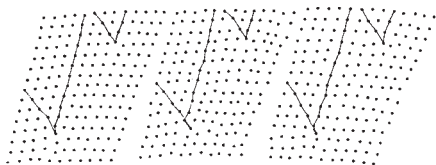


Fig. 2 The mosaic of blue cones (left), horizontal cells (middle) and the terminals of one bipolar cell type (right) in a small area of a fish retina. These populations, and others in the retina, are exactly matched, as seen from the congruent packing faults in each array, indicated by linked rows of cells.

varied composition of their clones. As in the *Drosophila* eye, and in the vertebrate central nervous system generally, the various cell populations are completed in sequence. In some species this process can result, as in the fly, in exact stoichiometry between the intricate receptor and neural cell populations formed, leading to near-crystalline tissue regularity (Fig. 2). Possibilities for instructive interactions between new and old cells can be seen in the half-formed structure of the rat retina at birth. While the inner retina, including most of the interneurons and glia of the inner nuclear layer, is complete, the outer part still comprises a uniform thick layer of ventricular precursor cells¹⁰.

Using the viral marker, Turner and Cepko analysed a large number of clones infected shortly after birth in rats. As expected, these clones were small (on average about two cells) and contained principally rods. About a quarter of early clones, however, contained one or two neurons or glia in the inner nuclear layer, in addition to labelled rods. It was these that varied in different clones, most frequently comprising bipolar cells, and less often amacrine cells or Müller glia, in no fixed combinations. These results form the evidence that, within the established broader inside-to-outside progress of retinal histogenesis, detailed sequences of cell division *per se* play no direct part in determining the identities of the many types of neural cells produced.

One should be alert, however, to two imponderables discussed by the authors. First, there must be gaps in the clonal ranks, arising from cell death, which continues in all retinal layers well after birth. One estimate is that 3–8 per cent of cells die, but this is based on guessed persistence times for dying cells, and may be too low. It appears unlikely, however, that there is scope left at this late stage for random deletions to conceal underlying

clonal patterns. The numerous one-cell clones must result mainly because provirus integrates into one of two possible founder cells at mitosis. The second imponderable is the possibility of cell-specific impairment in expression of the β -galactosidase gene. The authors argue that the wide variety of cell types labelled is some assurance against this, but equally, between many clones, integration at different locations in the host genome might evade net impairment statistically.

It is curious that this beautiful study, developing and applying an elegant new lineage marker, should be rewarded by evidence that lineage patterns are inconsequential in the system studied. But this has happened before, as seen, and had its own consolations. In any case, applying

the method earlier, and elsewhere in the nervous system, and introduction of other genes, promises much for the future. □

1. Turner, D.L. & Cepko, C.L. *Nature* **328**, 131–136 (1987).
2. Price, J., Turner, D. & Cepko, C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 154–160 (1987).
3. Raff, M.C., Miller, R.H. & Noble, M. *Nature* **303**, 390–396 (1983).
4. Raymond, P.A., Reifler, M.J., Rifkin, P.K. & Clendening, B. *Soc. Neurosci. Abstr.* **12**, 118 (1986).
5. Ito, K. & McGhee, J.D. *Cell* **49**, 329–336 (1987).
6. Sulston, J. & Horwitz, R. *Dev. Biol.* **56**, 110–156 (1977).
7. Lawrence, P.A. & Green, S.M. *Dev. Biol.* **71**, 142–152 (1979).
8. Hafen, E., Basler, K., Edstroem, J.-E. & Rubin, G.M. *Science* **236**, 55–63 (1987).
9. Banerjee, U., Renfranz, P.J., Pollock, J.A. & Benzer, S. *Cell* **49**, 281–291 (1987).
10. Blanks, J.C. & Bok, D.J. *J. comp. Neurol.* **174**, 317–328 (1977).
11. Ramon y Cajal, S. *Arch. Anat. Physiol.*, 400 (1893)

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Ocean Drilling Program

Glacial history of Antarctica

Leg 113 shipboard scientific party*

OCEANOGRAPHIC and climatic evolution over the past 66 million years (Myr) consists largely of progressive cooling and glaciation of the polar regions and perhaps some warming in the tropics, increasing thermal gradients across the Earth's latitudes. The climatic and glacial evolution of Antarctica is crucial to our understanding of long-term climatic change. High latitudes are among the most sensitive to externally driven change, and powerful feedback mechanisms are involved, related to ice albedo and bottom-water formation. Further light has been cast on these issues by the latest leg of the Ocean Drilling Program.

Leg 113 addressed in particular the following questions. When did the Antarctic ice sheets first form, and have they since been permanent? How did surface- and intermediate-water temperatures change in response to Antarctic glacial development? When did marine glacial conditions develop sufficiently for initiation of Antarctic bottom water formation in the Weddell Sea? How did planktonic productivity in the Weddell Sea sector of the Southern Ocean evolve, and is this development linked to the evolution of Antarctic climate and the oceanic environment, particularly the polar front?

These questions are answered in part by studies of sediments that contain fossil remains. During Leg 113 we drilled nine sites, representing a wide range of sedi-

mentary environments (Fig. 1). These sites were chosen to recover open-ocean (pelagic) sediments on Maud Rise (sites 689 and 690), hemipelagic and terrigenous (land origin) sediments on, or close to, the continental margins of East and West Antarctica (sites 691–693 and 695–697) and a deep-sea sequence from the Weddell abyssal plain that includes fine-

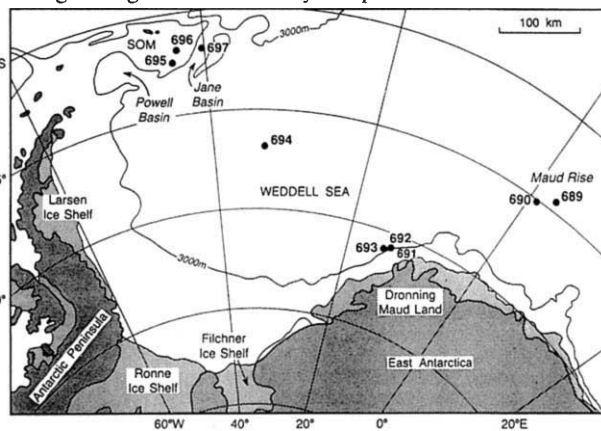


Fig. 1 Locations of Leg 113 sites in the Weddell Sea. All sites lie in the present-day Antarctic water mass, south of the polar front. SOM, South Orkney Microcontinent.

grained hemipelagic sediments and sand-rich sediments deposited from the turbidity currents (turbidites) (site 694).

Drilling results from these sites provide a rich database on the environmental history of Antarctica and the adjacent ocean from the early Cretaceous (120 Myr ago) to the present. The high organic content of the sediments from the early and middle Cretaceous (before 88 Myr) indicates that they were deposited under oxygen-free or oxygen-deficient conditions, possibly during times of fluctuating surface-water salinity. These environments were similar to those on the

Falkland Plateau at the same time. By the late Cretaceous (73 Myr) open-ocean sediments were being deposited on Maud Rise.

We found diverse planktonic assemblages suggesting that the surface-water near Antarctica was relatively warm from the late Cretaceous to the Eocene (58 Myr). East Antarctica was then a rich source of clay, which became dominated by smectite and kaolinite (associated with chlorite) during the Palaeocene (66–58 Myr). This result suggests that the climate was warm, that the humidity was increasing and that the continent was probably unglaciated. Eocene clays also are dominated by smectite, indicating a continuation of warm climate and a predominance of chemical over physical weathering processes. Eocene pollens at site 696 indicate the presence of temperate beech forests with an undergrowth of ferns on the northern Antarctic Peninsula. An abundant collection of diverse calcareous microfossils suggests that the ocean was nearly saturated in CaCO_3 and probably warm.

Sediments from the Cenozoic epoch, that spans 66 Myr to the present, reflect a sequential cooling of the Antarctic water mass, probably related to the glaciation of Antarctica. On Maud Rise, siliceous microfossils progressively replaced carbonate ones during the Cenozoic, with initial siliceous sedimentation at the Eocene/Oligocene boundary (37 Myr) becoming almost completely siliceous by the late Miocene (6–9 Myr). More recent sediments recovered from East and West Antarctic margin sites nearly all contain mainly siliceous microfossils. The change suggests that the water was probably highly unsaturated in CaCO_3 , and cool, even in the shallow waters of site 696 (present depth, 650 m). On Maud Rise, illite became an important clay during the early Oligocene (37–30 Myr), and smectite decreased, suggesting that there was strong cooling and/or an increase in aridity in East Antarctica at that time that reduced the extent of hydrolysis. But smectite continued to dominate at site 696 until the middle Miocene (16–10 Myr) because of the strong influence of the West Antarctic climate.

Oligocene sequences (37–24 Myr) drilled on the West Antarctic continental margin are not of sufficiently high quality to give details of glacial development, but give no indication of a major build-up of ice. There is little ice-rafted detritus in the Upper Oligocene sediments on Maud Rise. These fragments are present at site 693, however, indicating that there was

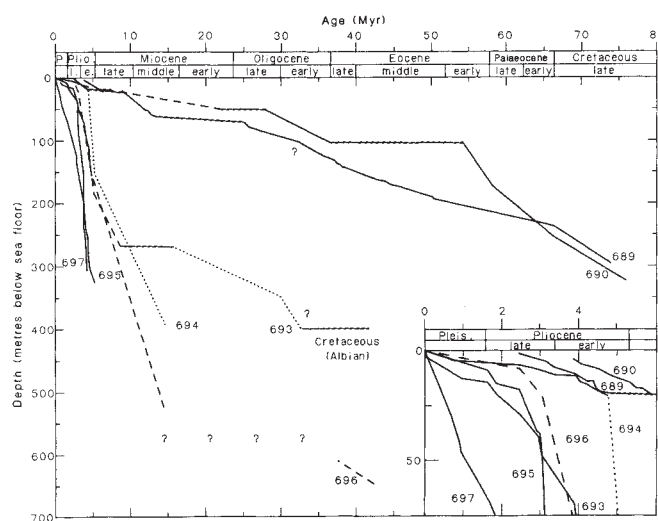


Fig. 2 Comparison of sedimentation rates at all Leg 113 sites. Solid lines, good chronostratigraphic control; short and long dashed lines, questionable intervals (possible slumped or barren intervals); horizontal wavy lines, hiatuses. The lower right-hand inset is an expanded graph for the upper 75 m of each site.

some ice on East Antarctica during the early Oligocene, the earliest found at any of the sites in the Weddell Sea. Reworked (displaced) sea-bed microfossils from around the Oligocene/Miocene boundary (24 Myr) at site 693 reveal that part of the continental shelf was shallow and at least partly ice free. Similar freshwater microfossils from the same era at site 696 suggest that there were freshwater lakes on the northern Antarctic Peninsula. At the same time there was a pronounced increase in the number of siliceous organisms relative to calcareous organisms at Maud Rise (sites 689 and 690), perhaps because of the growing extent of cool surface waters.

A further step in Antarctic evolution took place during the middle Miocene (16–10 Myr). At site 693, the middle Miocene record is missing in a hiatus and an increase in ice-rafted detritus follows in the sediments immediately above the hiatus. A significant cooling probably occurred during this time in West Antarctica as well, while smectite was being replaced by illite and chlorite at site 696. This indicates that strong physical weathering, probably because of climatic cooling, developed much later here than in East Antarctica. The absence of ice-rafted detritus throughout this interval at site 696, however, suggests that severe glaciation had not yet begun on West Antarctica.

Our data suggest that the West Antarctic ice sheet did not form until the late Miocene (10–5 Myr). This tentative conclusion is based on sedimentary evidence from site 694, the distribution of reworked planktonic microfossils, and changes in clay minerals. Samples from site 696 exhibit an increase in hemipelagic sedimentation, the presence of an abundant ice-rafted component and yet further

increases in concentration of illite and chlorite during the late Miocene. The West Antarctic ice sheet could have been unstable during the early stages of its development, indicated by the variability of sediment and the presence of a coarse component at site 694. The highest rates of turbidite deposition at this site ($>180 \text{ m Myr}^{-1}$; Fig. 2) may have occurred in $< 0.5 \text{ Myr}$ at the Miocene/Pliocene boundary (5.3 Myr). These high sedimentation rates possibly resulted from an expanding, unstable, West Antarctic ice sheet and occurred during an interval marked elsewhere by strong cooling, increased and very variable ^{18}O isotope concentrations and low sea level.

Palaeomagnetic data show that by the early Pliocene (4.8 Myr), turbidite deposition had virtually ceased at site 694, suggesting that the West Antarctic ice sheet became a permanent, stable feature. Sedimentation rates increased markedly at most sites at this time (Fig. 2), because of increased hemipelagic deposition and biosiliceous productivity. The past 2.4 Myr were characterized by markedly lower sedimentation rates (Fig. 2), decreased diatom abundances and poor microfossil preservation. This seems to represent another step in glacial intensification and an expansion of sea ice.

The Pleistocene (1.6 Myr) is marked by continued low sedimentation rates, intervals of abundant *Neoglobobulimina pachyderma* (a calcareous planktonic foraminifer) and calcareous benthic foraminiferal assemblages at all sites in the Weddell Sea shallower than about 3,400 m. The high rates of hemipelagic deposition at site 697 in Jane Basin during the Pleistocene reflect the continued transport of fine-grained sediment down the West Antarctic margin and possibly a stronger northwards flow of Antarctic bottom water. □

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Organic dust in comet Halley

SIR—In a recent article by Kissel and Krueger¹ a statement is made to the effect that the hypothesis of biological grains has been disproved for the organic dust from comet Halley as analysed by Vega 1. This conclusion turns out to be contingent on several assumptions that are artificially introduced into the data analysis. First, the implicit assumption that all of the cometary dust in our model² is biological may be incorrect, for even under optimal laboratory conditions a 20 per cent conversion of nutrient into cells would be considered an excellent yield. Second, cometary dust particles colliding with the mass spectrometer at speeds of 70 km s⁻¹, corresponding to an energy of 300 eV per carbon atom, could surely not be observed in anything like their pristine state. Impact ionization, dissociation and subsequent recombinations would have the effect of considerably modifying initial chemical structures. Attempts to reconstruct original molecular configurations using theoretical considerations involve uncertainties to a major degree. Furthermore, the differing distributions of ionization states for break-up ions corresponding to such elements as P, Na and K cannot be determined to any degree of precision, thus leading to uncertainties in estimates of elemental ratios. Finally, any estimate of the distribution of elements between the presumptive 'chondritic core' and a mantle is an artefact of modelling not necessarily connected with the properties of cometary dust. Model-dependent discriminatory criteria such as these cannot be used in logic as disproof of any particular model.

Perhaps more significantly the only data relating to pristine grains from comet Halley were derived not from expensive satellite experimentation but using ground-based telescopes such as the

Anglo-Australian Telescope. The figure shows the infrared data for the dust from this comet observed on 30 March 1976 compared with the predictions of the bacterial model³.

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1. Kissel, J. & Krueger, F.R. *Nature* **326**, 755–N. (1987).
2. Hoyle, F. & Wickramasinghe, N.C. *Living Comets* (University College Press, 1985).
3. Wickramasinghe, D.T. & Allen, D.A. *Nature* **323**–. (1986).

KISSEL AND KRUEGER REPLY—We would like to make some remarks concerning our article on the organic component in the dust from comet Halley¹. As we have already communicated elsewhere², the different distribution states of ionization of the atomic ions (such as Na⁺, K⁺, P⁺) after impact of a dust particle are well known within a factor of two over the periodic table. With these ion/atom yield ratios one recognizes the mineral parts of the grains as being, on average, chondritic, and the organic parts not containing phosphorous and with only ~ 10⁻⁶ sodium and potassium, with lines that may even be due to target contaminations only, as we have recently confirmed. Together with the coma gas measurements³, the total elemental distribution (of gas, mineral and organic dust) fits the solar distributions⁴ well; from this we are even more convinced about the validity of our atomic ionization distribution. But we admit that this was not the scope of the paper cited by Hoyle and Wickramasinghe; therefore we have decided to write a more comprehensive article⁵ about the methods and results of the elemental and anorganic composition.

Freeze-dried cells should contain at least a percentage of sodium and 0.1 per cent of potassium, which is clearly not the

case with the organic grains. Even the anorganic mineral parts contain less than 3×10⁻³ Na, less than 1×10⁻⁴ K, and less than 5×10⁻⁴ P. The organic part contains less than 10⁻⁵ P, which is not sufficient to form sufficient nucleic acids and phospholipids.

We never claimed that the large molecules possibly present in the organic part of the dust survive the impact process; however, those organic fragment ions that show up in the mass spectra tell us about the nature of the main chemical classes being present. As we find highly reactive states apparently with hydrogen and water driven out of the molecules, these do not fit the requirements of living cells, however, in warm water the prerequisites of self-replicative units may form, as known from Orgel, Lohrmann, and co-workers⁶.

We are, indeed, very astonished that one may draw conclusions of living matter being present merely from infrared spectra. Instead, we feel this to be a contingent way of treating empirical data. However, more detailed interrelationships between grain size, composition, and structure with infrared and mass spectra are known today⁴.

Finally, we regard the disproval of the concept of 'evolution' to be even more difficult after the analysis of the cometary dust by space-borne instruments.

J. KISSEL

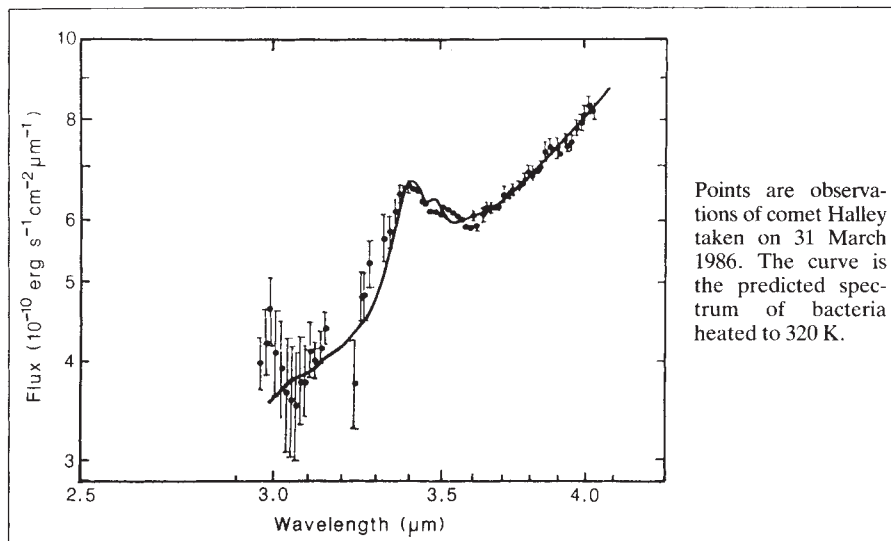
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1. Kissel, J. & Krueger, F.R. *Nature* **326**, 755 (1987).
2. Jessberger, E.K., Kissel, J., Feghtig, H. & Krueger, F.R. in *Physical Processes in Comets, Stars and Active Galaxies* (eds Hillebrandt, W. et al.) 26 (Springer, Berlin, 1987).
3. Krankowsky, D. et al. *Nature* **321**, 326 (1986).
4. Greenberg, J.M. et al. *Proc. Workshop on Fluffy Structures in Leiden, Netherlands*, May, 1987 (submitted).
5. Krueger, F.R. & Kissel, J. (manuscript in preparation).
6. Lohrmann, R., Bridson, P.K. & Orgel, L.E. *Science* **208**, 1464 (1980).

Body size, energy use and ecological dominance

SIR—Brown and Maurer¹ recently claimed that large species were energetically more important in communities than small species, in contrast with earlier findings^{2,3}. Their evidence is not compelling. Only 5 out of 39 of the bird and none of the fish regressions are statistically significant. Significance is incorrectly claimed for the mammal slope ($r = 0.56$, $n = 9$, therefore $P > 0.1$); only the plant assemblages are consistently statistically significant. More seriously there is a strong correlation between the slopes of their regression lines and the corresponding correlation coefficients (birds: $r = -0.76$, $P < 0.001$). The estimated slope for a perfect correlation is -1.34 (95% limits are between -1.07 and -1.61), strong evidence for the greater im-



Points are observations of comet Halley taken on 31 March 1986. The curve is the predicted spectrum of bacteria heated to 320 K.

portance of small species. In addition to these statistical inadequacies, counting bias in bird censuses is likely to underestimate slopes. Although a great range of abundances of small species will be recorded, large species will, in general, be represented by few individuals or recorded as present densities < 1 individual per census area. Species in the latter category are usually omitted from regression analyses, thereby artificially raising slopes.

A similar analysis to that of Brown and Maurer for pelagic ecosystems⁴ found slopes ranging between -0.77 and -1.18 , with medians close to -1.0 . However, this analysis used total densities, summed over all the species within a size class. To test if data treatment affected the conclusions I carried out both species density/species mass (Sp) and total density/size class mass (C1) regressions for the first 24 censuses reported in Griffiths⁵. For perfect correlations the estimated slopes of Sp and C1 regressions were -0.95 (95% limits -0.60 and -1.29) and -1.14 (-0.84 to -1.44) respectively. These estimates are not significantly different from each other or the estimate from the data of Brown and Maurer. Furthermore the slopes of Sp and C1 regressions for each census are strongly correlated with each other ($r = 0.83$, $P < 0.001$). Hence species/mass and total individuals/mass regressions both indicate that small organisms are energetically more important than large ones.

Harvey and Lawton⁶, in a News and Views comment, pointed out that (even if Brown and Maurer were correct) the total importance of small organisms was probably greater than that of large because there are usually relatively more small species than large in communities. The similarity of the Sp and C1 slopes casts doubt on this: there are approximately equal numbers of large and small species per logarithmic size class.

Analysis of insect samples from a variety of tropical habitats^{7,8} gives a median abundance/length slope of -3.4 (range -1.53 to -4.24 , $n = 18$). Hence small insects are as or more important than large ones, depending on the mass-length relation used⁹, consistent with an earlier analysis of some of these data^{10,11}.

Although most data suggest that small species and organisms are energetically at least as important as large ones this might not always be true. The biomasses of large mammals in tropical habitats¹² are more than an order of magnitude greater than those of small mammals¹³ (small mammals $28\text{--}1,500$, median 84 kg km^{-2} , $n = 9$; large mammals $405\text{--}19,928$, median $4,387 \text{ kg km}^{-2}$, $n = 24$). Because large and small mammals cover approximately the same relative size range these figures are directly comparable.

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BROWN AND MAURER REPLY — Virtually all of Griffiths' comments on our recent paper¹ are either incorrect or misleading. We make four points.

First, the slope of the regression between log population density and log body mass that we reported for mammals is not significantly different from zero, but it is significantly different from the values (-0.67 to -1.0) claimed in other studies^{2,3}.

Second, our data on birds do indeed show a significant correlation between the values for the slopes and the correlation coefficients. This is to be expected, because the correlation coefficient can be expressed mathematically (ref. 4, equation 15.7) as the slope multiplied by the ratio of the standard deviations of the two variables. This hardly invalidates our analyses. There is no justification for assuming, as Griffiths does, that the best estimate of the slope can be obtained by extrapolating this relationship to a perfect negative correlation ($r = -1.0$). To extrapolate slopes and correlations beyond the range of the data is a serious abuse of regression analysis⁵. In making this extrapolation Griffiths apparently assumes that all variation in population density or energy use among species of a given body mass can be attributed to measurement error. If there is significant error in the estimation of the two variables, a correct procedure would be to use some form of Model II regression. We showed that reduced major axis regression gave results similar to the Model I regressions that Griffiths criticizes. The wide variation in population density among organisms of similar size is undoubtedly real. Because this variation is heteroscedastic, we cautioned against fitting any linear relationship to these data and performed a more robust nonparametric analysis on the large data set for birds. This gave the same qualitative result: energy use per species is greater for large than for small birds.

Third, like comparable previous analyses^{2,3,6} that had reached opposite

conclusions, we considered only how energy use per species varied with body size. Harvey and Lawton⁷ and Griffiths raise the very different, but equally interesting question of how the collective energy use of all species within a logarithmic body size category varies with body mass. Our as yet unpublished analyses for birds are remarkably similar to those of Strayer⁸ for freshwater zooplankton, showing that total energy use for all species in a size class first increases and then remains approximately constant as body mass increases.

Finally, Griffiths' letter and the analysis of Peters⁶ suggest that log population density per species scales with log body mass, with a slope of -1.0 . This implies that energy use per species varies inversely (slope -0.25 to -0.33), and biomass per species is constant (slope $= 0.0$) with respect to body mass. Although our analyses suggest the contrary, we concede that this might be true within some taxonomically defined communities. It cannot be true for all organisms or entire ecological communities because that would require that the biomasses of all species-specific parasites and symbionts equal those of their hosts. Obviously not only biomass but also energy use per species of many kinds of small organisms must be much less than that of the rarest large ones.

We stand by our claim that energy use per species increases with body mass within local communities of many different kinds of organisms. Furthermore, even though there are often more species and higher population densities of small organisms than of large ones, total energy use of all species within logarithmic size classes probably does not decrease with increasing size.

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1. Brown, J.H. & Maurer, B.A. *Nature* **324**, 248–250 (1986).
2. Damuth, J. *Nature* **290**, 699–700 (1981).
3. Peters, R.H. & Wassenberg, K. *Oecologia* **60**, 89–90 (1983).
4. Sprules, W.G. & Munawar, M. *Can. J. Fish. aquat. Sci.* **43**, 1789–1794 (1986).
5. Griffiths, D. *Am. Nat.* **127**, 140–166 (1986).
6. Harvey, P.H. & Lawton, J.H. *Nature* **324**, 212 (1986).
7. Janzen, D.H. & Schoener, T.W. *Ecology* **49**, 96–110 (1968).
8. Janzen, D.H. *Ecology* **54**, 659–686 (1973).
9. Schoener, T.W. *Ann. ent. Soc. Am.* **73**, 106–109 (1980).
10. Morse, D.R. *et al. Nature* **314**, 731–733 (1985).
11. Denness, B. *Nature* **318**, 391 (1985).
12. Fleming, T.H. in *Small Mammals: their Productivity and Population Dynamics* (eds Golley, F.B., Petruszewicz, K. & Ryszkowski, L.) 269–298 (Cambridge University Press, 1975).
13. Coe, M.J., Cummings, D.H. & Phillipson, J. *Oecologia* **22**, 341–354 (1976).

1. Brown, J.H. & Maurer, B.A. *Nature* **324**, 248–250 (1986).
2. Damuth, J. *Nature* **290**, 699–700 (1981).
3. Peters, R.H. & Wassenberg, K. *Oecologia* **60**, 89–90 (1983).
4. Sokal, R.R. & Rohlf, F.J. *Biometry* 572 (Freeman, New York, 1981).
5. Gunst, R.F. & Mason, R.L. *Regression Analysis and its Application* 12–15 (Dekker, New York, 1980).
6. Peters, R.H. *The Ecological Implications of Body Size* (Cambridge University Press, 1983).
7. Harvey, P.H. & Lawton, J.H. *Nature* **324**, 212 (1986).
8. Strayer, D. *Oecologia* **69**, 513–516 (1985).

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

Rewards and fairies

S.A. Barnett

Upon Further Reflection. By B.F. Skinner. Prentice-Hall: 1987. Pp.214. \$24.95, £21.70.

ONE OF the many American admirers of Burrhus Frederic Skinner has written: "Virtually every institution in the United States has been touched by behavior modification, if only in feeling a need to erect defenses against it". Behaviour modification here stands for the application, in schools, factories and clinics, of Skinner's neobehaviourism, in which all human conduct, intelligent, moral and aesthetic, is reduced to the consequences of previous rewards and punishments. The actions of a human being reading Plato, learning to play the piano or writing a book on psychology are interpreted in the same terms as those used for a rat in a "Skinner box" pressing a lever to release a pellet of food. In such experiments, movement of the lever, called the operant, is usually the only event recorded.

The book under review consists of 14 essays, published in the 1980s, each of which could evoke a lengthy comment. The title suggests a change of mind or at least a response to criticism; and Skinner does declare, on good grounds, that "psychology as a science is . . . in a shambles" (p.160) — a state of affairs for which he might accept some responsibility. His remedy, however, is to return to the analysis of *behaviour* and the external circumstances, or contingencies, that influence it — the research programme that dominated American psychology for several decades. His principal enemy is "cognitive" psychology, about which he is amiably rude. This discipline, he says, has abandoned natural science and "rejoined the philosophers in the study of mind, language, values, and perception" (p.160).

Self-knowledge, we are told, is of the same kind as the knowledge others have of ourselves, "about stimuli, responses, and consequences" (p.105). If so, presumably when one says one is hungry or in love, one is making an inference from one's behaviour, observed as if from outside.

The objections to neobehaviourism do not, however, depend on introspection. Among the major phenomena that fall outside Skinner's system are spontaneous exploring and innovation. An animal or human being may move around an unfamiliar region, with no incentive other than that of experiencing novelty. Such movement promotes learning which can be used later to improvise a new, adaptive route or detour. Yet Skinner writes:

"Early man . . . did not look at pictures or listen to music . . . When he had nothing to do, if we may judge from related species, early man simply slept or did nothing" (p.26). This passage fits the author's presumptions, but not the facts. When their primary needs are satisfied, both human beings and other mammals commonly display a restless curiosity;

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B. F. Skinner — a bleak portrait of *Homo operans*.

and the ill effects of depriving people of stimulation are well known. The longing for Nirvana is not a universal human instinct.

Among the leading examples of improvisation are those of speech. Almost every long sentence we utter is new. Young children devise their own neologisms and ungrammatical but intelligible sentences. To state that such achievements are the result of "contingencies of reinforcement" does not provide even a useful description, let alone a causal analysis. Skinner's comments on language reveal his unstated metaphysical presumptions with unusual clarity. "As I have argued in *Verbal Behavior*, thinking can be adequately formulated simply as behaving. A sentence is not the expression of a thought; it is the thought" (p.87). Here Skinner brushes aside the independent human being who creates new sentences and ideas. Only study of external stimuli and the responses to them, he declares, can provide authentic knowledge of human action; only they are proper material for a scientific psychology; and

above all, the external stimuli determine what we do.

In these essays, however, the determinism is reinforced by evolutionary theory. "Human behavior", he writes, "is the product of (1) the contingencies of survival responsible for the natural selection of the species and (2) the contingencies of reinforcement responsible for the repertoires acquired by its members" (p.55). The evolution of culture is a by-product: "A culture may be defined as the contingencies of social reinforcement maintained by a group" (p.74). Natural selection is presented as an axiom: the absorbing question of defining it, and the problems of relating the evolution of

Homo sapiens to current theory, make no appearance.

Skinner's bleak portrait of *Homo operans* — a puppet controlled by genes and rewards — seems to give him and us no scope for trying to improve the human condition. Yet the first essay is entitled "Why We Are Not Acting to Save the World". If this is anything more than Skinner responding to contingencies of reinforcement, then it is a statement of what we *ought* to be doing. Indeed, Skinner asks: "Rather than wait for further variation and selection to solve our problem, can we not *design* a way of life that will have a better chance of a future?" (p.8). We are perhaps entitled to wonder whether our difficulties are partly because of our liking for over-simple stories about complex issues, or because for decades we have been forcefully told that we are mindless creatures, without wills of our own, whose actions are biologically pre-programmed. Possibly, as a result, some of us are reduced to a state of inert resignation or despair.

Not that Skinner intends to depress us. But he advocates, as always, only "improving the strengthening contingencies of behavior" (p.27), especially in education, therapy and industry. "Classical remedies, such as letting the worker have a greater share in decision making, do not attack the central problem, which lies in the contingencies" (p.29). The massive evidence in favour of such remedies is not rebutted or even mentioned. We see again that the autonomous person does not exist in the neobehaviourist's cosmos.

The author, as many of these essays show, is a person of marked good will, even though his doctrine disallows his will as an influence on his actions. His benignity, the simple lucidity of his message, the charm of his writing and the fame of his teaching will ensure that he has many readers. I wish I could say confidently that they will find the book rewarding. □

The author, as many of these essays show, is a person of marked good will, even though his doctrine disallows his will as an influence on his actions. His benignity, the simple lucidity of his message, the charm of his writing and the fame of his teaching will ensure that he has many readers. I wish I could say confidently that they will find the book rewarding. □

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Testing times in Australia

John Simpson

A Very Special Relationship: British Atomic Weapon Trials in Australia. By Lorna Arnold. HMSO, London: 1987. Pp. 323. Pbk £7.95.

OVER the past two years, the nuclear trials conducted by the British government in Australia between 1952 and 1963 have been the subject of an Australian Royal Commission and several books (see the review in *Nature* 318, 319; 1985). Lacking, however, has been a concise and balanced account of these activities which attempts to place them in their historical context, rather than judge them from the perspective of current values and political issues.

Lorna Arnold now offers us such a study, but at the same time gives us a foretaste of some elements of the forthcoming official history of the British nuclear programme during this period. This makes her book of interest not only to those who wish to know more about the tests in Australia, but also to those who seek to understand something of the nature of the development of nuclear weapons. Arnold has had access to British documentation on both the Australian tests and the nuclear-weapon programme, and has interviewed many of its managers. The result is a very readable and informative volume.

One problem with writing books about nuclear tests is that a full understanding of each test demands knowledge of the weapon research and development programme being attempted. Yet details of such programmes have been the most closely guarded of state secrets. The originality and strength of Arnold's account is that she has been allowed to lift a corner of the veil of secrecy still surrounding the British programme in the 1950s, and offer an authoritative but limited explanation of what the Australian tests were for.

The book does not cover the Christmas Island tests in 1957 and 1958, and the picture is therefore far from complete. But it does contain some previously unrevealed elements. These include the three weapon types which were under development in late 1955; the use of enriched uranium and boosting in weapon tests in late 1956; the revised testing objectives of July 1956; the three weapons other than Blue Danube that Britain was in a position to produce by mid-1957; the emphasis, by 1957, on the development of small-yield plutonium warheads for use on anti-aircraft missiles and as a smaller and lighter trigger for H-bombs; the testing in 1957 of advanced fission devices with

mixed plutonium and uranium cores; and the need by 1959 to contemplate safety tests on nuclear weapons being made in Britain to Anglicized American designs. This type of information gives coherence to Arnold's account, and enables a much clearer picture to be built up of why specific tests were conducted. It also enables some evaluation to be made of the significance of the tests.

In the course of her account Arnold deals in a low-key manner with almost all of the recent accusations that have surrounded the issue of testing in Australia. She accepts several of them, and offers explanations of why others should be rejected. This is therefore not an apologia on behalf of the British government, although she maintains throughout that levels of exposure to radiation were within acceptable limits.

Many readers will no doubt regard Arnold's book as suspect on at least two counts: much of the documentation she has used is not yet available for independent evaluation, and she follows the official line that exposure to low levels of radiation carries no appreciable health risk. Yet despite some minor errors which suggest the book was rather hurriedly completed, it remains a convincing, broad-based and fair account of the events in Australia three decades ago. As a result of its publication, the general public will be able to make its own informed judgement on the actions of the British and

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Cloud over Maralinga — fireball of the nuclear device exploded at Maralinga, South Australia, on 9 October 1957. It was the fifteenth such test carried out by Britain.

Australian governments of the period, and of the decisions taken by the managers of the British nuclear-testing programmes. □

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Not lurid virology

Beverly E. Griffin

DNA Tumor Viruses: Control of Gene Expression and Replication. Edited by Michael Botchan, Terri Grodzicker and Philip A. Sharp. Cold Spring Harbor Laboratory: 1986. Pp. 620. Pbk \$75.

BOOKS, even scientific ones, used to be for reading. One author (or occasionally two), one book. Thus among the books that influenced me in my choice of career were well-written and thought-provoking tomes such as Fruton and Simmonds's *General Biochemistry*, Watson's *Molecular Biology of the Gene*, and Luria's *General Virology* (billed to me by my bookseller as *Lurid Virology*). Those in other disciplines would, I suspect, come up with similar lists of one- or two-author volumes.

DNA Tumor Viruses, Vol. 4 in Cold Spring Harbor Laboratory's *Cancer Cells* series, contains a wealth of information, but for all that — or because of all that — it is not designed to be read. More than 200 authors were involved, and I suspect that it will not have much influence on anyone's career. The editors say that one of

their objectives was to update the 1980 book of the same title, but the latter, known to many simply as "Tooze", had one editor and 14 authors. It was meant to be read, and potentially was therefore not without influence on a part, at least, of the scientific community.

I dwell at length on this subject because I am beginning to wonder about the role of books and whether authors, editors (and publishers) have much concern for the reader, or whether he or she, like the dodo, is becoming an anachronism. Maybe the current trend in which books increasingly mimic journals (with editors as referees), and in which journals proliferate beyond a recognizable readership, has gained too much momentum to be halted. If it spreads, will even novels be written by multiple authors? I hope not.

I am, of course, being unfair to the book under review. But having taken the preface at face value, I was disappointed at the failure of many of the contributors to deliver. In addition to the *raison d'être* given above, the editors promise a history of DNA tumour viruses followed by an elaboration of current and future developments in the field. In general, the second aim is paid lip service only. One of the great frustrations for me was to find that Dulbecco, a scientist greatly valued for his

contribution to research on DNA tumour viruses, and one who certainly could have remedied this situation, had spoken on the occasion that gave rise to the book, and had neither contributed to it nor had his talk included. We are told only that Dulbecco "discussed the future of molecular biology"! Further, the occasion was deemed (as judged from pictures included in the book) to be a tribute to Joe Sambrook's role in the development of Cold Spring Harbor as a centre for the study of DNA tumour viruses, but, surprisingly, there is no contribution from him.

The "overviews", which take up about a quarter of the book, consist of four uncoordinated and stylistically variable chapters on SV40, polyoma, papilloma, adeno and herpes viruses. These are viruses with some claim to a cancer association, cancer being broadly and loosely defined. The chapter by Broker and Botchan on "retrospectives and prospectives" of papilloma viruses is very good indeed, but the others fail to measure up to this standard. They mostly give the impression that the authors couldn't see the wood for the trees. These chapters — the promised update of "Tooze" — are followed by over 50 contributions from a variety of laboratories, with headings such as "Transcription", "Regulation of Transcription", "Processing and Translation", "Transformation" and so on, that attempt to provide the threads for linking together often quite disparate data, some novel, some raw and some rewarmed. The overall emphasis is on adenoviruses, undoubtedly reflecting the interests of Cold Spring Harbor Laboratory and the scientists associated with it. Here is it probably salutary to remind ourselves that the main clinical features of this virus are the respiratory infections, pharyngitis and conjunctivitis. That aside, some of the basic science presented, particularly on adenovirus, is very elegant, and the short article by P.A. Sharp, "Analysis of Splicing of mRNA Precursors *In Vitro*", alone makes it worth continuing as far as page 245. Much of the information given in the book appears in primary journals, however.

Overall, this discourse on DNA tumour viruses is a helpful catalogue of events and a good source of references. I can admire the occasional flashes of insight it provides. Its failures reflect the fact that scientists these days are generally "too busy" to pay much attention to the more global aspects of their work. Likewise editors. Further, like any work that focuses more on data than ideas, it will become rapidly dated. I was left to wonder why the publications department at Cold Spring Harbor Laboratory did not consider asking Tooze to update "Tooze". □

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Putting humanity in context

Ian Tattersall

Another Unique Species: Patterns in Human Evolutionary Ecology. By Robert Foley. Longman:1987. Pp. 313. Pbk £12.95. To be published in the United States in December by Wiley, provisional price \$32.95.

It is lamentable, if hardly surprising, that as our egocentric species has laboriously compiled its own autobiography over the past century or so, it has tended to depict itself not simply at the centre of the evolutionary stage but as occupying virtually the entire proscenium. One is thus refreshed by the title of Robert Foley's new book, *Another Unique Species*. Here is a work on human evolution that starts by calling attention to the fact that *all* species are unique, so our much-vaunted feeling of apartness simply serves to align us with the rest of nature in yet another way.

Inevitably, though, because Foley is concerned here with the evolutionary history of *Homo sapiens*, our species is perforce once more the star of the show. But then, that is in the very nature of autobiography, and indeed what gives this genre its peculiar fascination. What the author actually manages to do is to squeeze various other parts of nature into our story in something a little better than the few familiar cameo roles: the herbivores that were there to be hunted; the plants that were there to be gathered; the rocks that were waiting to be picked up, carried around and fashioned into tools.

Foley's purpose is, of course, rather broader than this: to integrate the story of early human evolution into the wider picture of the changing African biosphere, and to show that throughout their evolution humans have been subject to the same environmental constraints as other vertebrate species. He attempts to do this by examining the evidence, such as it is, for climatic and faunal change in Africa over the past several million years, and applying to it certain principles of evolutionary ecology, which might be characterized more accurately as a set of rules-of-thumb derived from empirical comparisons made across the modern fauna.

One problem that immediately arises is that, while what evidence we have indicates that climatic oscillation has clearly been the rule over the period in question, Foley's approach to evolution is strictly neo-darwinian and (despite his protestations) equally strictly adaptationist. There is thus a tension throughout the book

between the relatively short-term ecological fluctuations Foley describes, and the necessarily long-term putative evolutionary responses by hominids with which he is clearly most comfortable. This is true even though he makes the crucial but generally overlooked observation that conditions promoting population fragmentation and coalescence — and hence hominid speciation and interspecies competition — were rife in the Plio-Pleistocene. Laudably, though, he is not constrained by his view of speciation as a special case of anagenesis from speculating that the African Plio-Pleistocene harboured many more hominid species than are currently recognized.

Rapidly improving though it is, the human fossil record is rather too sparse to allow testing of most of the predictions Foley makes from his ecological principles. This results in a discussion that in the main is reminiscent of the just-so stories about human evolution that the rest of us have been telling for many years. The process does however throw up some interesting speculations — for instance that meat-eating among hominids may have begun in the context of seasonal dietary stress; or that the robust australopithecids had a relatively specialized feeding strategy that was most successful in times of maximum species richness, while the most generalized *Homo* assumed the advantage in times of deteriorating environmental quality.

My main difficulty is in deciding for whom the book was written: it is obviously intended to be accessible to students, and offers background information clearly aimed at them. But it does not contain enough discussion of the human fossil record, or of palaeoenvironments, or of ecology to serve as a text in any of these areas. Perhaps it will find a niche as supplemental reading in general palaeoanthropology courses. □

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New in paperback

- *The Nemesis Affair: A Story of the Death of Dinosaurs and the Ways of Science* by David M. Raup. Publisher is W.W. Norton, price is \$6.95 (the book comes out in Britain in October, price £5.95).
- *The Age of Birds* by Alan Feduccia. Publisher is Harvard University Press, price is \$12.95, £10.50. For review see *Nature* 290, 646 (1981).
- *Perception and Communication* by D.E. Broadbent, with a new foreword by Michael I. Posner. Publisher is Oxford University Press, price is £15, \$26.95. For review see *Nature* 182, 1,572 (1958).
- *Power Production: What Are the Risks?* by J.H. Fremlin, with a new postscript on the accident at Chernobyl. Publisher is Oxford University Press, price is £6.95. For review see *Nature* 316, 583 (1985).

Before Bakewell

Julian Wiseman

Like Engend'ring Like: Heredity and Animal Breeding in Early Modern England. By Nicholas Russell. *Cambridge University Press*: 1986. Pp.271. £27.50, \$42.50.

THE agricultural revolution in Britain, which gathered momentum during the eighteenth century, was characterized in part by the rise in use of cropping techniques based upon sophisticated and flexible rotations of roots, grasses and legumes. Improved cropping provided more fodder, which in turn encouraged livestock husbandry. The additional manure that was produced increased soil fertility, and the process was complete — thereby breaking the vicious circle of declining crop yields, impoverished soils and limited raising of livestock typical of farming in mediaeval times.

During the preceding 200 years there had been some interest in animal breeding, but it was generally confined to species of military or social value, principally the warhorse and, later, the thoroughbred. Although farm animals were of undoubted economic importance, they came lower down the animal hierarchy and were largely ignored by professional breeders and chroniclers. But there were developments in their breeding ("improvement") before the late 1700s and the emergence of Robert Bakewell, the Leicestershire farmer often regarded as the founder of modern breeding practice. In this well-researched, stimulating and informative book, Nicholas Russell seeks to illuminate the work of Bakewell's forerunners.

It was not until the late 1600s that writers on breeding broke free of the influence of classical texts. These were often the inspiration for Renaissance authors, and contemporary debates on reproduction were in consequence mixtures of philosophy, astrology and mythology, and were singularly unhelpful to the practical breeder. The concept of heredity was ill-defined, certainly quantitatively, and the confusing effects of environment on animal structure and form was an added complication. If the appearance of stock could be dramatically altered merely by changing feed inputs, then parental roles in influencing sibling characteristics must have seemed haphazard and unpredictable. Location was considered an important determinant of breed appearance which, it was thought, could thus be altered simply by moving stock from one region to another.

There was too a fashion for anthropomorphic features such as "character" and "beauty". Russell cites writers on breeding

in early modern England who attempted to relate the latter to more readily identifiable features, including coat colour. Markham, however, a Tudor, considered ancestry, pedigree and lineage to be more important, and the breeding of the thoroughbred horse and the origins of the stud book owed much to such opinions. Anomalies arising from this obsession — many thoroughbreds were demonstrably useless and full siblings often dissimilar — were conveniently ignored.

Breeding policy for cattle and sheep frequently followed similar patterns, although most practical graziers were concerned more with numbers fattened than appearance and lineage, suggesting that selection was not a high priority. Rudimentary attempts to improve breed output for these animals were recorded early in the eighteenth century, particularly in those lowland regions of the Midlands already regarded as innovative — in the uplands, survival rather than improvement was the issue. Heterogeneity of stock was troublesome, and farmers often inbred their cattle and sheep in an attempt to concentrate quality. This

unfortunately led to degeneration and reinforced the notion of pedigree as a key determinant of quality.

Robert Bakewell's prominent role in the development of animal breeding is undoubted, but Russell argues that the evidence that his animals were biologically superior is dubious, citing their poor fecundity and excessive carcass fat — views which verge on the heretical amongst many modern animal breeders but which accord with the limited information available. Bakewell, however, was an extremely able farmer. He identified the importance of economic and biological criteria in judging an animal's value, as opposed to subjective assessment of fancy points. Despite his reservations, Russell concludes that Bakewell can be regarded as a pioneer in the popularization of the idea of animal output as a key to selection policy. Agriculture's loss is that such ideas were not incorporated into general breeding policy until well into this century. □

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Mixed messages

Peter Brimblecombe

Arctic Air Pollution. Edited by B. Stonehouse. *Cambridge University Press*: 1987. Pp.328. £30, \$49.50.

MANY people still see the Polar areas as being the Earth's final frontiers. This may explain some of the public's anxiety over pollution of these distant and uninhabited regions. But while it is true that the air of the Arctic and Antarctic is no longer pristine, little public attention is drawn to the extremely low level of contamination or to the point that, as yet, air pollution around the Poles appears to have had no effect on human health or the sensitive ecosystems.

Still, pollutants *are* reaching the remotest parts of the Earth. It is appropriate that governments, such as the State of Alaska, support research and sponsor conferences like that held in Cambridge, UK, in September 1985. This book is one result of the symposium. The thoughtful reviews can be read with profit by students and scientists generally concerned with the environment. Many of the papers stray well beyond the confines of Polar pollution; there are reviews of low-level exposure to ionizing radiation, indoor pollution and source apportionment by trace element enrichment in aerosols, for example, that will stimulate and inform those with no particular interest in the Arctic at all.

Naturally, the important research using

ancient ice to establish long-term trends in atmospheric composition receives ample attention. Chapters on the climatic implications make it clear that much work needs to be done to understand the effects of Arctic haze and blackened snow on the radiation balance of the Earth. I was particularly fascinated by C. S. Benson's argument that the habitation haze of Fairbanks, Alaska, is a new type of urban air pollution, differing significantly from the smoke-fogs of London and the photochemical smogs of Los Angeles. While seemingly unable to show any significant effects in the Arctic, a number of papers on the health and ecological issues are excellent accounts of their subjects.

In the introduction, Professor Glenn Shaw advises us against being over-pessimistic about Arctic air pollution. However some authors still seemed to feel the need to make bold comments about the situation in their particular area of study. Statements such as "... only international cooperation and speedy resolution will lessen the social costs of Arctic haze" serve to confuse lay people and fuel the spectacular media stories that so frustrate scientists in Polar research.

However this disagreement with the mood of some of the reviews is very much a personal judgement. The book itself is a high-quality production, which feels good to hold and bears a striking grainy cover photograph. And it contains a remarkably good collection of articles. □

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Unpleasant surprises in the greenhouse?

Wallace S. Broecker

There is now clear evidence that changes in the Earth's climate may be sudden rather than gradual. It is time to put research into the build-up of carbon dioxide in the atmosphere on a better footing.

THE inhabitants of planet Earth are quietly conducting a gigantic environmental experiment. So vast and so sweeping will be the consequences that, were it brought before any responsible council for approval, it would be firmly rejected. Yet it goes on with little interference from any jurisdiction or nation. The experiment in question is the release of CO₂ and other so-called 'greenhouse gases' to the atmosphere. Because these releases are largely by-products of energy and food production, we have little choice but to let the experiment continue. We can perhaps slow its pace by eliminating frivolous production and by making more efficient use of energy from fossil fuels. But beyond this we can only prepare ourselves to cope with its effects.

The task of scientists is to predict the consequences of the build-up of CO₂ and other gases. To be useful these predictions must be reasonably detailed, but we are in no better a position to make them than are medical scientists when asked when and where cancer will strike a specific person. Understanding the operation of the joint hydrosphere – atmosphere – biosphere – cryosphere system is every bit as difficult as understanding the factors that determine whether or not cancerous cells will get the upper hand. Because of our lack of basic knowledge, the range of possibility for the greenhouse effects remains large. It is for this reason that the experiment is a dangerous one. We play Russian roulette with climate, hoping that the future will hold no unpleasant surprises. No one knows what lies in the active chamber of the gun, but I am less optimistic about its contents than many.

My suspicion is that we have been lulled into complacency by model simulations that suggest a gradual warming over a period of about 100 years. If this seemingly logical response to a gradual build-up of greenhouse gases is correct, then one can imagine that man may be able to cope with the coming changes. While I do not have any complaints about how these modelling experiments were conducted — indeed they were done by brilliant scientists using the best computers available — the basic architecture of the models denies the possibility of key interactions that occur in the real system. The reason is that we do not yet know how to incorporate such interactions into models.

My impressions are more than educated

hunches. They come from viewing the results of experiments nature has conducted on her own. The results of the most recent of them are well portrayed in polar ice, in ocean sediment and in bog mucks. What these records indicate is that Earth's climate does not respond to forcing in a smooth and gradual way. Rather, it responds in sharp jumps which involve large-scale reorganization of Earth's system. If this reading of the natural record is correct, then we must consider the possi-

"We play Russian roulette with climate [and] no one knows what lies in the active chamber of the gun . . ."

bility that the main responses of the system to our provocation of the atmosphere will come in jumps whose timing and magnitude are unpredictable. Coping with this type of change is clearly a far more serious matter than coping with a gradual warming.

For more than 100 years scientists have been aware that the Earth is in the midst of a series of cyclic glaciations. Oxygen isotope measurements made on microscopic shells from deep-sea sediments provide a continuous record of these events. They show that cycles averaging 100,000 years in length carried us from warm climates, comparable to today's, to the cold climates of full glacial time. Power spectra for these records point a finger at cycles in the Earth's orbital characteristics as the driving force^{1,2}. Seasonality was at times stronger and at times weaker than it is today. Although most scientists now accept the orbital hypothesis, none of the mechanisms that have been proposed to explain the linkage between seasonality and climate is universally accepted. This matter is the subject of much research.

The ¹⁸O/¹⁶O record in deep-sea cores gives the impression that the response of the climatic system to orbital forcing is smooth and gradual (Fig. 1 — see over). Only recently have we begun to realize that this impression is a false one. One of the early clues came from studies of the ecology of the remains of planktonic organisms contained in deep-sea cores of rapidly accumulated sediment from the North Atlantic³. The ecological changes recorded in these cores probably reflect changes in surface-water temperature.

This record does not show the gradual change scientists had become accustomed to. Instead it shows an abrupt end to glacial time and, even more interesting, a brief period of intense cold interrupting the warm period that followed (Fig. 1). Although the two records shown in Fig. 1 are quite different, they are not incompatible. Changes in ¹⁸O/¹⁶O in the shells of marine sediments are largely the result of the waxing and waning of the ¹⁸O-deficient continental ice caps. As the response time of global ice caps is thousands of years, the ¹⁸O record smooths out the rapid changes in climate.

It took more than this, however, to make us take these abrupt changes seriously. The evidence that turned our heads came from holes drilled through the Greenland ice cap. As a foot or so of ice forms from each year's snowfall, the record captures changes in the ice-cap environment no matter how rapid they have been. These changes are recorded in the ratio of isotopically 'heavy' to isotopically 'light' water in the ice (a measure of air temperature)^{4,5}, in the content of particulate matter in the ice (a measure of the dustiness of the air over the ice cap)⁶ and in the content of CO₂ and other greenhouse gases contained in the air trapped as bubbles in the ice (a measure of the atmosphere's greenhouse capacity)⁷. Like the ecological record in deep-sea muds from the North Atlantic, the ice core records from Greenland give a dramatically different impression of the manner in which climate changes than do the oxygen isotope records for marine shells. They show that during glacial time climate changed frequently and in great leaps. The typical leap involves a 6°C change in air temperature, a fivefold change in atmospheric dust content and a 20% change in the CO₂ content of air. In cold times the air was dustier and contained less CO₂.

While the record in Greenland's ice shows that climate can change in big leaps, we have to look further for a clue as to why these leaps occur. The last of the events seen in the Greenland record is well documented not only in Greenland ice and in North Atlantic sediment, but also in lake and bog sediments from throughout Western Europe and maritime Canada⁸. On land it is recorded by large shifts in the ecology of plants (as recorded by their pollen grains). During warm periods trees grew in these areas; during cold periods

the trees were replaced by tundra shrubs. Although this last of the Greenland events is found in bog and lake sediments throughout northern Europe, it is not seen in similar records from the United States. This geographical distribution suggests the North Atlantic Ocean as the culprit. Based on this clue, we are beginning to see how devilish are the links between components of the climatic system.

Rather than responding smoothly to gradual forcing, the ocean-atmosphere system appears to respond by changing the way it works. India's climate provides an apt analogy. The winters in India are very dry because of the descent of cold air from the Tibetan plateau. Summer heating causes this atmospheric circulation pattern to reverse abruptly. Air rises from the Tibetan plateau, drawing oceanic air across the Indian subcontinent, and the dry conditions give way to monsoonal rains.

The Earth's climatic system currently works in a way beneficial to northern Europe⁹. This region is warmed by heat released from the surface waters of the North Atlantic. The amount is a staggering 30% of that received by the North Atlantic from the Sun! This heat is steadily carried northward, as on a conveyor belt, by the ocean circulation system. In the vicinity of Iceland the warm water meets cold air. The air warms and ameliorates climate on the adjacent land. The water cools, sinks to the abyss and flows as a great river, down the full length of the Atlantic, around Africa, through the southern Indian Ocean and finally up the Pacific Ocean (Fig. 2). This current carries 20 times more water than the world's rivers combined.

In the North Pacific the ocean conveyor belt runs just the other way round. Deep waters move towards the north and rise to the surface and then flow towards the Equator in the upper ocean. So in today's world, the Atlantic conveyor belt carries tropical heat for delivery to the atmosphere at high northern latitudes, while the Pacific conveyor belt forces cold surface waters to move southward, pushing the invading warm waters back towards the Equator.

Why does our ocean operate in this fashion? Although we don't have the complete answer, we do have the first principle. The circulation system is governed by salt. Because of the difference in the circulation patterns, surface waters in the North Atlantic are on average warmer than those in the North Pacific. This allows more water to evaporate from the North Atlantic than from the North Pacific, and in turn gives rise to a net transport of water vapour through the atmosphere from the Atlantic to the Pacific. The North Atlantic is enriched in salt by this process (and the North Pacific waters correspondingly diluted). The enrichment of

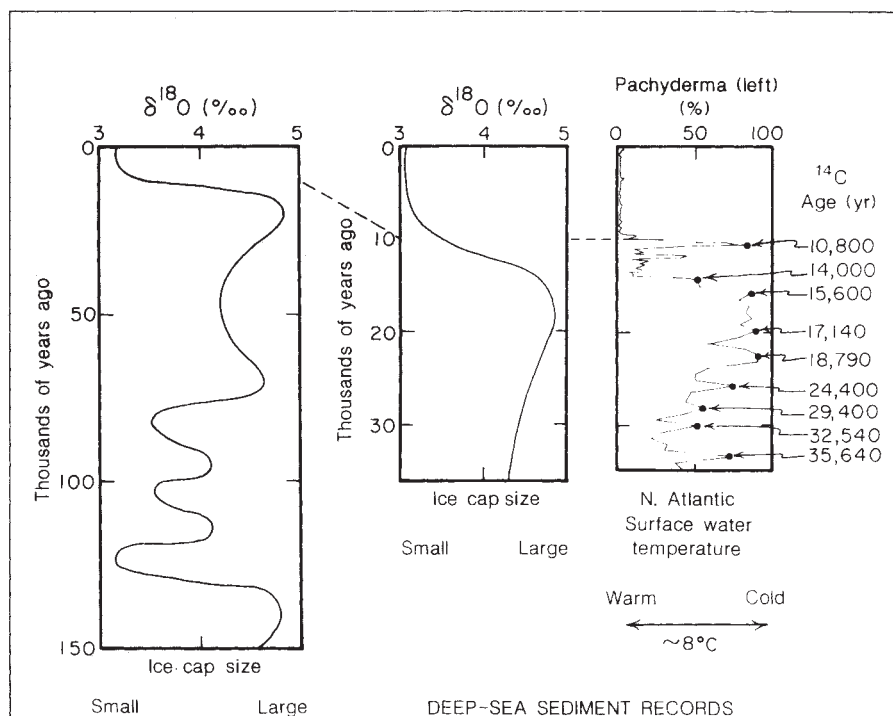


Fig. 1 The ratio of heavy oxygen (^{18}O) to light oxygen (^{16}O) in foraminifera shells preserved in deep-sea sediments provides our best record as to how the great continental ice sheets of the Northern Hemisphere waxed and waned. The reason is that the snow that builds ice caps is depleted in the heavy oxygen isotope. Therefore as ice caps grow, the ocean is correspondingly enriched in heavy oxygen. So also is the oxygen in the calcium carbonate of shells living in these waters.

The oxygen isotope record on the left covers just over one major climate cycle. There have been eight such cycles in the past million years. The diagram in the middle is an enlargement of that on the left, designed to emphasize that these oxygen isotope records indicate a gradual and smooth transition from full glacial to full interglacial conditions. Recent findings demonstrate that this view of natural climate change is misleading. The sluggish retreat of the ice and blurring of the record by burrowing worms combine to give what proves to be the wrong impression.

On the right is the record from a deep-sea sediment obtained west of the British Isles. This record is based on the abundance of the shells of the planktonic herbivore *Pachyderma* (left coiling) which lives in waters adjacent to Greenland. It is the only species of planktonic foraminifera found in these icy waters. During cold periods this species dominated the entire North Atlantic, providing up to 90% of the shells found in the sediments off Britain.

As can be seen, this record tells a different story. The North Atlantic warmed abruptly about 15,000 years ago, and has so remained (except for two brief cold relapses) until the present. It is the abruptness of the warming and of the two brief and intense epochs of renewed cold that are of great interest. The second of these cold epochs was first recognized from study of the pollen records in bogs created in northern Europe during the retreat of the last great ice sheet. These records show that the forests which repopulated this area after ice departed were destroyed by a brief and intensely cold interval of several hundred years' duration. They were replaced by the shrubs of glacial time.

salt in the North Atlantic must somehow be compensated by a flow of salt through the sea from Atlantic to Pacific. The compensation in today's ocean is achieved by the flow of a deep current of salty water from the Atlantic to the Pacific and a matching flow of correspondingly less salty water around the other way in the upper ocean.

The phenomenon that maintains this situation is, I suspect, a potentially dangerous one. The circulation pattern is self-reinforcing and hence self-stabilizing. The deep current is driven by the extra density supplied to the waters of the North

Atlantic by the enrichment of salt. The enrichment of salt is driven by the heat carried by the warm water which flows northward in the upper Atlantic to supply the deep current. A classic chicken and egg situation! Excess evaporation causes the deep current; the deep current causes excess evaporation.

What are the consequences of perturbation of this system? Palaeoclimatic evidence points to a shutdown of the North Atlantic conveyor belt during glacial periods. Such a shutdown would cool the North Atlantic and its adjacent lands by $6-8^\circ\text{C}$. This in turn would cause the boreal

forests in these areas to give way to tundra shrubs. The sparsity of vegetation would permit far more dust to be lifted into the atmosphere. Finally, the only feasible mechanisms scientists have come up with to explain rapid changes in the atmosphere's CO_2 content involve modifications in the ocean's circulation pattern and intensity. Thus we surmise that the reorganization of ocean circulation that accompanied the shutdown of deep-water production in the North Atlantic produced all of the environmental changes recorded in ice cores. The many leaps in climate seen in the glacial part of the Greenland ice core records probably represent flips of the system back and forth between two self-stabilizing modes of operation.

It must be emphasized that the leaps in climate seen in the ice and sediment records for the North Atlantic region were confined to the cold parts of the climatic cycle. Following the transition from cold to warm conditions 10,000 years ago, the climate in this region has remained remarkably constant. Apparently Earth's climatic system has remained firmly locked in its present mode of operation. If so, what is the likelihood that increases in CO_2 and other greenhouse gases will jolt the ocean-atmosphere system out of its current mode into one more suitable to the coming conditions?

Unfortunately, we have little basis for answering this critical question. Nature provides us with no recent analogy to the super-interglacial conditions we are about to generate. The climate of the past 10,000 years is representative of the warmest part of the last several glacial cycles. Hence nature has not explored the super-interglacial climatic regime (at least not in sufficiently recent times that our geological records give an adequate picture of Earth's environment to be useful for future prediction). Further, as we have only recently become aware of the complexity of the linkages which tie together ocean, atmosphere, ice and terrestrial vegetation, we have not even begun to formulate means by which these linkages might be modelled. Indeed, reliable modelling may never be possible.

If we are to get into a position to cope with the effects not only of the greenhouse gases, but also of the various poisons being released to the environment, we must greatly expand our efforts to understand the individual parts of the Earth's great surface system and the interactions among them. The task, however, is every bit as complex as that of preventing cancer.

I see no hope of getting much done in the United States without a fundamental reorganization of our approach to environmental research. I believe that most scientists would agree that the handling of research on greenhouse effects by the Department of Energy and on acid rain by

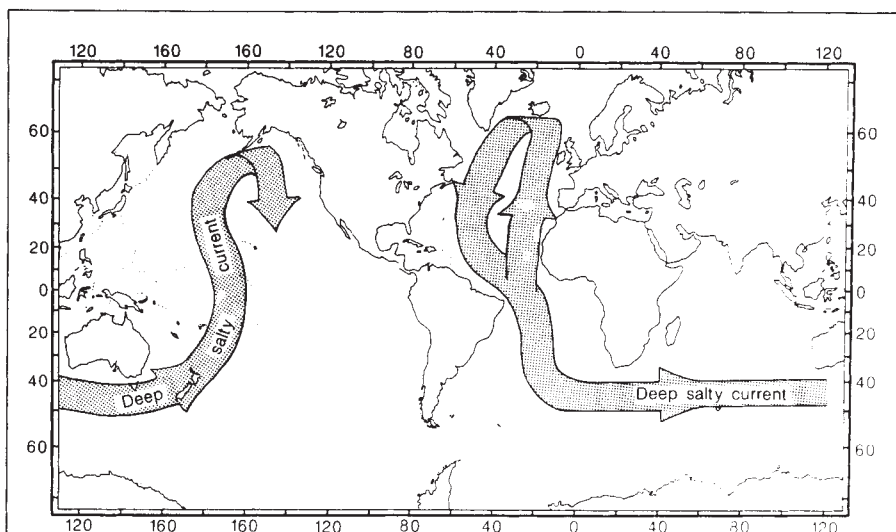


Fig. 2 A large-scale salt transport system operates in today's ocean to compensate for the transport of water (as vapour) through the atmosphere from the Atlantic to the Pacific Ocean. Salty, deep water formed in the North Atlantic flows down the length of the Atlantic, around Africa, through the southern Indian Ocean and finally northward in the deep Pacific Ocean. Some of this water upwells in the North Pacific, bringing with it the salt left behind in the Atlantic due to vapour transport. This atmospheric water vapour and ocean salt transport system is self-stabilizing. Records from ice and sediment tell us that this great conveyor system was somehow disrupted during glacial time and replaced by an alternative mode of operation.

the Environmental Protection Agency has been ineffective. The problem lies partly in the perception by the managers of these programmes that their mandate is to obtain quick fixes, and partly in their inability to grasp the subtleties of the problems placed under their jurisdiction. The result is that these managers avoid long-term strategies designed to build up our base of knowledge. Rather, they are strongly attracted by proposals to re-work existing information even though most scientists agree that it has been squeezed nearly dry. Thus, while the existing programmes serve to keep management informed, they do little to further our ability to predict the consequences of our actions. It is as if the efforts to prevent cancer were based largely on epidemiological information with no biochemical or cellular research. If we are to get out of this rut, the responsibility for basic research on environmental systems must be wrested from these mission-orientated agencies and given instead to an organization which is dedicated to basic research and is isolated from immediate political pressures.

As it now stands, a healthy component of basic research is being carried out only in those areas where there is a strong tradition of federal sponsorship (that is, the atmospheric and ocean sciences). Research on the continental parts of the environmental system (vegetation, soils and waters) remains in the Dark Ages. It's a Catch-22 situation — managers at the National Science Foundation (NSF) feel that basic research in these areas should be sponsored by the appropriate mission-

orientated agency, but such agencies shun basic research.

Unique opportunities to expand our knowledge of environmental systems slip by without being fully exploited. A prime example is the invasion of man-made tracers (that is, nuclear-testing tritium and radiocarbon, reactor radiokrypton and industrial freons) into the sea. A detailed knowledge of the patterns and rates of movement of these substances through the sea will prove a tremendous boon to the validation of general circulation models for the atmosphere-ocean system. Although the atmospheric side of such models is fully developed, the oceanic side is still in a primitive state. Further development is hampered by our lack of knowledge of the physics of key processes occurring in the sea. Because of this, ocean models of adequate sophistication are decades away. Despite the obvious value of tracer data, the efforts to map the passage of these substances through the sea's interior are sparse. NSF is concerned that surveying brings too little immediate scientific yield, while the mission-orientated agencies fail to appreciate the fact that if we are to understand the Earth's system we must adopt long-term strategies. With the exception of the French, who have developed a remarkably good tracer programme in the Indian Ocean, other industrial countries have shown no greater interest in ocean tracers than had the United States. Hence future ocean modellers will rue the deficiencies in the documentation of man's great ocean-tracer experiment.

I am not suggesting that things could

be altered simply by increasing NSF's budget. NSF is not set up to put strings on the use of its funds. Nor should it be. But to do the kind of research that will be required if we are to deal effectively with long-term environmental problems, there must be some strings.

In this time of budget deficits it may be unrealistic to call for new government entities, but I am convinced that the only way to straighten out the mess in environmental research is to create a new national institute. This institute would have two objectives. First, it would sponsor environmental research; second, it would advise the government on a broad range of environmental questions.

The International Committee of Scientific Unions has recently given its blessing to a new initiative in environmental science, the International Geosphere-Biosphere Program (IGBP). The aim is to further our understanding of the operation of the individual elements of the environmental system and of the linkages between them. The mission-orientated agencies in the United States are already planning how to capture their share of this new pie. Judging by past performance, it would be a mistake to let them have any of it. Instead, the US monies to be spent on IGBP should be funnelled through a new entity, The National Institute for the Environment. The primary responsibility of this institute would be to develop integrated programmes dedicated to furthering our knowledge of environmental systems. In particular, the Institute would focus on those areas that most need attention, namely, the continental components of the climatic system. The programmes would be planned by the most capable scientists in the country and conducted in the most appropriate laboratories (university, industry and government). The institute would be overseen by a board of trustees, consisting of eminent scientists. Its director, chosen by this board, would be a highly capable scientist, skilled in management. While the institute would have its own in-house scientific staff, most of its resources would go towards the funding of external activities.

There are isolated success stories. For example, two NASA managers, Shelby Tilford and Bob Watson, are running a programme designed to determine the sources of atmospheric methane and how the strengths of these sources have changed with time. This programme is a beautiful example of how environmental research should be conducted. It was designed by the nation's most knowledgeable scientists and is being carried out by highly capable groups throughout the country. The cost is modest, about two million dollars a year, and the payoff will be large. It works because Watson and Tilford are themselves outstanding scientists who know what the tractable problems are and

where the intellectual resources to tackle them reside.

Most of the research of the new institute should be housed in universities. It is in such settings that excellent research is generated for a minimum cost. By placing graduate education and research under one roof, an optimum environment for both is created. With a few exceptions, environmental research in large laboratories operated by government agencies has, in my opinion, proved to be unproductive and wasteful.

There are, however, two tasks that clearly require facilities not available to

"Not only do our current managers lack a proper intellectual grasp of the problem, but they are obsessed with legislatively imposed 'five-year reports', and give little attention to developing a long-term strategy to build the needed base of knowledge."

universities. One involves satellite observations, which will be the cornerstone of the effort to monitor the climatic system. Such platforms are best operated by NASA. The other is large-scale climate modelling, which is now the basis for all climate prediction. These facilities require large research staffs and immense computer power. Fortunately, a successful format has already been found which involves cooperation between universities and government departments. The National Oceanic and Atmospheric Administration has its primary effort on Princeton's campus. NASA bases such an effort on Columbia's campus. NSF operates such an effort at its National Center for Atmospheric Research in Boulder, Colorado. Because of the great importance of the so-called 'general circulation models' to climate prediction, these existing laboratories should be strengthened. Also, similar efforts must be launched in other university settings.

Five specific areas in which research must be greatly expanded can be singled out for special attention: (1) the large-scale circulation of the ocean; (2) the processes regulating soil moisture; (3) the processes responsible for cloud formation; (4) the role of biogeochemical processes on the atmosphere's composition; (5) the processes regulating sea ice.

Of course, there are already research programmes in these areas, but in my estimation they will not come up with answers quickly enough. In each area we need major new observational programmes to supply the key data that are necessary to develop a better physical understanding. In each area we need a cadre of young scientists with the appropriate training.

We also have to intensify our study of the climatic changes that have taken place over the past 100,000 years. As discussed above, nature herself has conducted large-scale climatic experiments. The response of the system to these natural experiments is recorded in sediments and in ice. By study of these records, we will be able to learn valuable lessons about the interactions that link the various elements of Earth's system together. I should stress once again that it is changes in these linkages which are likely to carry the greatest threats.

Although we don't know nearly enough about the operation of the Earth's climate to make reliable predictions of the consequences of the build-up of greenhouse gases, we do know enough to say that the effects are potentially quite serious. Whatever happens, it seems to me that the Earth's remaining wildlife will be dealt a serious blow. If, as the climatic record in ice and sediment suggests, changes in climate come in leaps rather than gradually, then the greenhouse build-up may threaten our food supply. To date, we have dealt with this problem as if its effects would come in the distant future and so gradually that we could easily cope with them. This is certainly a possibility, but I believe that there is an equal possibility that they will arrive suddenly and dramatically.

To prepare ourselves, we must take the problem of climatic change as seriously as we take those of cancer and nuclear defence. There are no easy solutions, and we must gear up for the long, hard job of working out how Earth's climate operates. To do this will require not only more financial and human resources, but also the administration appropriate to the task. Not only do our current managers lack a proper intellectual grasp of the problem, but they are obsessed with legislatively imposed 'five-year reports', and give little attention to developing a long-term strategy to build the needed base of knowledge. Even with a great intensification of effort, I fear that the effects of the rise in concentration of the greenhouse gases will come largely as surprises. But the greater our knowledge, the greater the wisdom that will be brought to bear if surprises do come. □

1. Hays, J.D. *et al.* *Science* **194**, 1121–1132 (1976).
2. Imbrie, J. *et al.* *Milankovitch and Climate Part I* (eds Berger, A.L. *et al.*) 269–305 (Reidel, Dordrecht, 1984).
3. Ruddiman, W.F. & McIntyre, A. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **35**, 145–214 (1981).
4. Dansgaard, W. *et al.* *Science* **218**, 1273–1277 (1982).
5. Dansgaard, W. *et al.* in *Green Ice Core: Geophysics, Geochemistry and the Environment* (eds Langway, C.C. *et al.*) *Am. Geophys. Un. Monogr.* No. 33, 71–76 (1985).
6. Hammer, C.U. *et al.* in *Green Ice Core: Geophysics, Geochemistry and the Environment* (eds Langway, C.C. *et al.*) *Am. Geophys. Un. Monogr.* No. 33, 90–94 (1985).
7. Stauffer, B. *et al.* *Ann. Glaciol.* **5**, 160–164 (1984).
8. Rind, D. *et al.* *Climate Dynamics* **1**, 3–33 (1986).
9. Broecker, W.S. *et al.* *Nature* **315**, 21–25 (1985).

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Thorium in G-dwarf stars as a chronometer for the Galaxy

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Observation of the radioactive nuclide ^{232}Th (half life 14 Gyr) in stars of various ages offers the possibility of directly relating the timescale for nucleosynthesis to that for stellar and galactic evolution. An initial set of such observations reveals no detectable evolution of the thorium abundance with respect to a stable element. This result is seen as evidence for a short timescale for galactic evolution and also for a problem with the stellar age scale.

STELLAR spectra usually provide only elemental abundances, but the element thorium has only one long-lived isotope, ^{232}Th , which has a half life comparable to the suspected age of the Galaxy. Its abundance relative to that of a stable element, therefore, might make a useful cosmochronometer. Observed in stars having age estimates based on stellar evolution theory, such a chronometer would provide information on the history of element synthesis, and perhaps also on the reliability of the stellar evolution timescale. The present study is an exploration of these possibilities.

The nature of the problem may be seen by considering a simplified galactic evolution model. Species i is produced according to a production function $P_i p(\tau)$, and is destroyed by radioactive decay with decay constant λ_i . Here $p(\tau)$ is a time-dependent function assumed valid for all species, and P_i is a constant giving the relative production of the particular species. The notation used closely follows that of Schramm¹. Nucleosynthesis is supposed to begin at a time $\tau=0$ in the Galaxy, and to continue until today at $\tau=T_0$. Once a star is born at time T , its outer atmosphere provides an unchanging sample of the general composition of the Galaxy at that time, modified only by the free decay of radioactive species. The differential equation governing the abundance of i is then

$$dN_i(\tau)/d\tau = P_i p(\tau) - \lambda_i N_i(\tau)$$

The abundance observed today in a star born at $\tau=T$ is, in this case, given by

$$N_i(T) = P_i e^{-\lambda_i T_0} \int_0^T p(\tau) e^{\lambda_i \tau} d\tau$$

which is a weighted integral of the production function up to stellar birth, followed by free decay until today. For a stable species s , $\lambda_s=0$, and the chronometer of practical interest is given by the ratio

$$\frac{N_i(T)}{N_s(T)} = \frac{P_i}{P_s} e^{-\lambda_i T_0} \int_0^T p(\tau) e^{\lambda_i \tau} d\tau \bigg/ \int_0^T p(\tau) d\tau \quad (1)$$

$$\approx \frac{P_i}{P_s} [1 - \lambda_i (T_0 - \langle \tau(T) \rangle)]$$

In the latter approximation, $T_0 - \langle \tau(T) \rangle$ is the mean age of the elements observed today in a star born at time T .

The oldest stars in this model will have $\langle \tau(T) \rangle=0$, and the youngest $0 < \langle \tau(T_0) \rangle < T_0$, depending on the history of synthesis. The existence of short-lived species in the early Solar System¹ indicates $p(T_0 - 4.6 \text{ Gyr}) > 0$. From the recent calculations² assigning ages of 16–18 Gyr to the oldest stars, it may be inferred that $T_0 \geq 18 \text{ Gyr}$. Observations of thorium in old and young stars could therefore yield interesting information on the shape of $p(\tau)$. If no evolution in the chronometer can be found, then either $\langle \tau(T) \rangle \approx 0$ for all T (essentially requiring a single synthesis event at the beginning), or T_0 must, in reality, be much less than

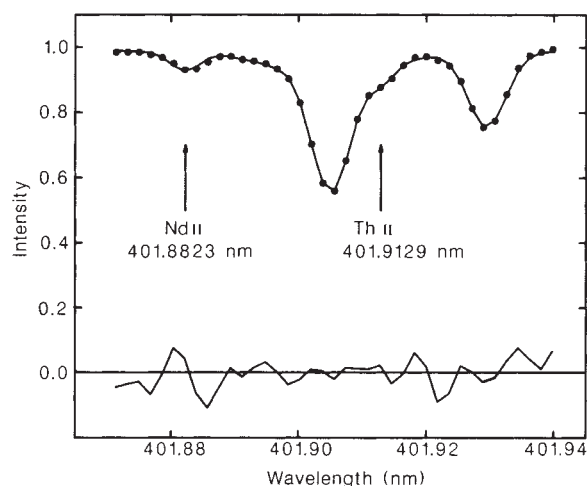


Fig. 1 Spectral data and fitted model for HR509. The survey lines of thorium and neodymium are marked. To within a constant, the ratio of the strengths of these lines is equal to the abundance ratio of the two elements. Filled circles are the stellar data; the solid line through the data is the numerically fitted model spectrum; and the solid line around zero is ten times the difference between the data and the model.

$\lambda_{\text{Th}}^{-1} = 20 \text{ Gyr}$ (so no significant radioactive decay has occurred in any star).

Observational strategy

Several faint absorption lines in the solar spectrum have been proposed as being due primarily to thorium³. The strongest of these, at 401.913 nm, originates from the ground state of the once-ionized atom and has an accurately measured transition probability⁴. The region surrounding this line in a solar-type spectrum is shown in Fig. 1. The line itself is located in the wing of a stronger feature, which is a blend of lines from neutral iron and nickel. A line due to ionized neodymium is found close by, at 401.882 nm, which has nearly the same strength as the thorium line. The neodymium line originates from a lower energy level only 0.06 eV above the ground state; its first ionization potential of 5.49 eV is also close to that of thorium at 6.08 eV (refs 5, 6). The two lines, therefore, will be from the dominant ion of each element over the regions of line formation in solar-type stars, and will follow nearly identical behaviour with temperature and gravity. It follows that the thorium to neodymium abundance ratio is directly proportional to the ratio of the line strengths. The proximity of the lines in wavelength should permit an accurate line strength ratio to be derived, sensitive to the placement of the stellar continuum level to second order only. There exist, in any case, two excellent continuum windows in the vicinity, at 401.866 nm and 401.967 nm, which are believed to be within $\sim 1\%$ of the 'true' continuum⁷.

Table 1 Stars studied spectroscopically

Star	Time	5,040/ T_e	log g	[Fe/H]	v_μ	$v \sin i$	Age	Refs
Sun	60	0.87	4.4	0.0	1.3	2.0	4.6 $\pm$ 0.1	
HR98	3,300	0.89	4.0	-0.2	1.3	3.0	10.0 $\pm$ 2.0	26, 29, 34-35
HR509	5,600	0.95	4.4	-0.4	0.8	0.0	9.0 $\pm$ 4.0	26, 29, 35-36
HR1136	3,000	1.05	3.9	-0.1	0.8	0.0	15.0 $\pm$ 4.0	26, 29, 35
HR3018	41,400	0.90	4.0	-0.8	1.3	1.0	19.0 $\pm$ 3.0	26, 29, 35
HR4523	3,600	0.90	4.1	-0.2	1.0	0.0	8.0 $\pm$ 3.0	33
HR4540	4,600	0.81	4.3	+0.2	1.3	2.0	7.0 $\pm$ 2.0	26, 29, 30-32
HR5072	9,900	0.92	3.8	-0.1	1.3	1.0	5.0 $\pm$ 3.0	26, 29-30, 32
HR5459	120	0.89	4.4	-0.1	1.3	2.0	6.0 $\pm$ 3.0	37-38
HR5460	1,300	0.99	4.5	+0.1	0.5	0.0	6.0 $\pm$ 3.0	37-38
HR5699	14,400	0.95	4.0	-0.5	1.0	1.0	15.0 $\pm$ 3.0	26, 29, 36
HR6060	14,400	0.88	4.2	0.0	1.3	2.0	3.3 $\pm$ 2.0	30-31
HR6585	4,800	0.89	4.2	+0.4	1.3	1.0	10.0 $\pm$ 2.0	26, 29, 36
HR6752A	12,600	0.95	4.5	-0.1	0.8	0.0	0.8 $\pm$ 0.8	32
HR7227	19,400	0.80	4.5	+0.1	1.3	3.5	0.6 $\pm$ 0.2	26, 29, 36, 39-40
HR7373	6,400	0.88	4.2	+0.3	1.3	0.0	9.0 $\pm$ 3.0	26, 29, 33
HR7665	8,600	0.91	4.4	+0.3	1.0	0.0	11.0 $\pm$ 5.0	26, 29
HR7896	7,200	0.90	4.0	0.0	1.5	3.0	1.0 $\pm$ 1.0	26, 29
HR1346	3,000	1.01	2.4	+0.1	1.3	0.5	0.6 $\pm$ 0.2	39-40
HR5058	3,600	1.08	2.4	—	1.3	0.5	—	
HR7869	2,000	1.08	2.4	—	1.3	0.5	—	

Column 1, identification numbers in ref. 26; the Sun refers to the spectrum of clear daytime sky.

Column 2, total integration time in sec.

Column 3, effective temperature parameter, T_e in Kelvins; from refs 41, 42, or from published photometry.

Column 4, log of stellar gravity in cm s^{-2} ; mostly from refs 41, 42, or estimated by myself.

Column 5, log of ratio of stellar to solar mean heavy element abundances, from refs 41, 42 except HR4523 and 7227, which have mean abundances of moving group.

Column 6, micro-turbulent velocity parameter in km s^{-1} .

Column 7, projected rotational velocity in km s^{-1} .

Column 8, age estimates and uncertainties in Gyr.

Column 9, literature references for age determinations.

The strategy adopted here is to measure the line strengths of these two weak lines, the ratio of which is nearly independent of stellar atmospheric structure, in a sample of stars having very similar atmospheres. The resulting line strength ratio, hereafter denoted Th/Nd, should be as accurately determined as the signal-to-noise ratio of the stellar spectra and the de-blending analysis of the thorium line will allow. Variation of the ratio with stellar age will be the chronometric quantity considered. Whether the observations will eventually yield a valid chronometer is *a priori* uncertain. The r-process (rapid) production ratio may vary with time and/or synthesis environment, and while thorium is produced only in the r-process, the abundance of neodymium is partially due to the s-process (slow)^{8,9}. An observational study such as this one is needed to shed further light on the matter. It is known that the ratios of r-process and s-process contributions do not vary among stars by more than about 25%, at least in stars with overall heavy element abundances more than a few per cent of solar^{10,11}.

Data acquisition

The data set studied here was obtained during two observing runs, 2-6 September 1983 and 3-7 April 1985, using the 1.4-m Coude Auxiliary Telescope and Coude Echelle Spectrometer¹² of the European Southern Observatory (ESO), Cerro La Silla, Chile. This extraordinary spectrometer is characterized by optimal spectral resolutions near $\lambda/\delta\lambda = 100,000$, high optical efficiency, and very low levels of scattered light. Equipped with an 1872-channel solid-state diode array (Reticon) detector, spectra adequate for the detection and study of thorium may be obtained from stars as faint as the sixth magnitude.

The sample of stars finally selected for analysis is given in Table 1. Also shown are the total integration times, usually broken up into several sub-integrations on different nights; stellar temperatures, gravities and heavy element abundances (in nearly all cases from the published literature); the assumed micro-turbulent and rotational velocities (as estimated from the

Table 2 Spectrum synthesis model

Line ID	Wavelength	Ref.	Multiplet	Solar W_λ
Nd II	401.8823	15	19	6.6
x0 II	401.8916	—	—	4.8
x1 II	401.8970	—	—	2.4
Fe I	401.9044	43	219	40.9
Ni I	401.9058	43	72	28.8
Th II	401.9129	44	3	7.4
x2 II	401.9190	—	—	2.2
x3 I	401.9279	—	—	8.3
Co I	401.9270 .175	16	16, 18	18.8
	.9279 .176			
	.9292 .313			
	.9394 .247			
	.9320 .042			
	.9332 .048			

Column 1, element and spectrum (ionization state).

Column 2, wavelength in air in nm; for Co also relative component strengths.

Column 3, literature reference for wavelengths.

Column 4, multiplet no. in RMT⁶.

Column 5, solar equivalent width in milliångstroms; from fit to Sun spectrum.

present data set); and the adopted stellar ages. The latter have been estimated in a variety of ways: position in the colour-magnitude diagram, membership in a moving group of stars having an age estimated from its colour-magnitude diagram, strength of Ca II emission and/or Li absorption lines, low heavy-element abundances characteristic of halo stars, and for the Sun the age given by radioactive decay in meteorites. For the old and intermediate age stars, the estimates have been put on the VandenBerg scale, using photometry, distances and composition estimates from the indicated literature sources and the isochrones in ref. 29. Error estimates are the writer's personal

judgement of the reliability of the age data, assuming the overall age scale to be correct.

Spectra of all the objects in Table 1 were obtained at a spectral resolution of 100,000 and a reciprocal dispersion of 0.0018 nm per detector channel. The total wavelength region covered by the observations was 400.2–403.5 nm, most of which has been used only to define the run of the continuum level and to calibrate the wavelength scale. Calibrations obtained were zero-signal integrations of various lengths, periodic integrations of a hollow cathode Th–Ar lamp for wavelength determination and regular measurements of an incandescent lamp to define channel-to-channel sensitivity differences. These calibrations were applied to the data using standard routines in the ESO IHAP software system¹³. With sufficient integration time, the detector system can deliver signal-to-noise ratios of 1,000:1; most of the stellar data here, however, have ratios in the range of 100 to 500:1. For the fainter stars, the final noise figure is set by particle event detections, which produce a markedly asymmetric noise distribution; for this reason, multiple integrations have been important in assessing and assuring the quality of the data.

Data analysis

Model spectra of the wavelength region between 401.87 and 401.97 nm have been calculated and compared to the observed spectra for each programme star. Line strengths and other parameters have been adjusted iteratively to achieve a good fit. Single-layer, homogeneous stellar atmospheres, which have the temperatures and gravities given in Table 1, have been assumed. Lines are assumed to be formed in local thermodynamic equilibrium (LTE) by pure absorption and to have line-to-continuum opacity ratios independent of depth in the atmospheres.

The lines included in the modelling are listed in Table 2. Line identifications have been assigned following previous discussions^{3,6,14}, with the faint features of unknown origin being the minimum set consistent with reasonable fits over the present data set. No good constraints on the identifications of the latter lines have resulted from the present study. The lines of neodymium and cobalt are known to exhibit isotope and/or hyperfine splitting; at the temperatures and turbulent velocities relevant here, the neodymium line may be modelled as a single component (ref. 15; and J. Blaise, personal communication), whereas the two cobalt transitions with their respective hyperfine structure components^{16–17} may be modelled by a single set of six components originating from a lower level at 0.58-eV excitation.

Calculation of the line absorption coefficients has been carried out according to standard practice^{18,19}. Unfortunately, the various line-broadening parameters cannot be satisfactorily fitted independently of each other over so short a length of spectrum. The procedure adopted has been to fix estimates for the shape of the instrumental profile; for the value of the so-called micro-turbulent velocity (describing turbulence on scales smaller than the photon mean free path in the stellar atmospheres); for the projected stellar rotational velocity, $v \sin i$; and for the (van der Waals) line damping width. The remaining width parameter is the macro-turbulent velocity (describing turbulence on scales larger than the photon mean free path); this width has been allowed to float during the iterating process.

Damping parameters for the iron line are available from Thomas–Fermi wavefunction calculations^{20,21} and have been scaled with stellar temperature and gravity^{19,20}. The damping width of this line is expected generally to be larger than those of average metal lines, and the damping widths of all other lines here have been (arbitrarily) set to 10% of that of the iron line. The iron line is found always to dominate the Fe–Ni blend, and only its wings should affect the Th/Nd ratio, irrespective of the values chosen for the damping of the other lines. Slightly better fits are obtained, however, using a damping width for the iron

Table 3 Numerical fitting results

Star	v_{macro}	Th	Nd	Th/Nd	Th/Nd ₀
Sun	1.1	7.4	6.6	1.15±0.08	1.10±0.08
HR98	1.6	7.6	7.9	0.97±0.06	0.93±0.06
HR509	1.0	6.9	4.9	1.50±0.08	1.12±0.06
HR1136	1.4	19.2	18.0	1.19±0.13	1.08±0.12
HR3018	1.6	3.6	2.7	1.36±0.28	1.30±0.27
HR4523	0.9	5.8	5.3	1.13±0.15	0.88±0.12
HR4540	1.7	7.7	7.3	1.08±0.07	1.04±0.07
HR5072	1.0	11.2	11.3	1.00±0.07	0.96±0.07
HR5459	1.0	9.1	9.2	1.00±0.10	0.96±0.10
HR5460	0.8	12.9	10.5	1.49±0.17	0.95±0.11
HR5699	1.3	4.8	4.6	1.06±0.19	1.02±0.18
HR6060	0.7	9.0	8.7	1.06±0.16	1.02±0.15
HR6585	1.5	9.5	10.0	0.95±0.11	0.91±0.11
HR6752A	1.2	9.9	8.2	1.31±0.12	1.07±0.10
HR7227	2.0	5.7	6.3	0.91±0.10	0.87±0.10
HR7373	1.4	13.6	12.4	1.13±0.16	1.08±0.15
HR7665	1.4	12.1	11.7	1.08±0.09	1.04±0.09
HR7896	1.4	14.3	13.1	1.13±0.10	1.08±0.10
HR1346	1.9	32.8	33.0	1.04±0.11	1.00±0.11
HR5058	1.1	32.6	66.8	0.16±0.03	—
HR7869	1.7	38.6	41.0	0.93±0.10	0.89±0.10

Column 1, star name from ref. 26.

Column 2, fitted value for macro-turbulent velocity, given v_{μ} and $v \sin i$ from Table 1, in km s^{-1} .

Column 3, equivalent width Th II 401.9129 nm from fit, in mÅ.

Column 4, equivalent width Nd II 401.8823 nm from fit, in mÅ.

Column 5, ratio of fitted line strengths.

Column 6, ratios in column 5 corrected for velocity width effect; see also Fig. 2.

line equal to one third of the theoretical value, and the final Th/Nd ratios reported in Table 3 have been derived using this value.

The final set of parameters fitted simultaneously to each data spectrum numbers twelve: the continuum height, the radial velocity of the star, the macro-turbulent velocity, and nine line strengths. The initial model of the spectrum of a star was determined by scaling the line strengths with temperature and gravity from the solar values, and by interactively setting a beginning radial velocity and macro-turbulent velocity. The subsequent nonlinear least squares fitting procedure²² calculated the various derivatives explicitly at each wavelength at each iteration. Convergence to a final fit generally required less than five iterations. Perhaps not unexpectedly, damping had to be applied to the radial velocity and macro-turbulent velocity parameters to stabilize convergence.

The typical quality of the resulting fits is seen in Fig. 1. The difference of the data and model spectra is, in each case, taken to be a measure of the noise in the fit, and is used to generate an estimate of the error of each parameter. For faint and weak-lined stars, the difference looks like random noise; for strong and narrow-lined spectra, spectrum modelling deficiencies are found to limit the achievable noise level. The final results of the fitting analysis are given in Table 3.

Some checks

Several specific checks for potential contamination of the programme lines, and also on the sensitivity of the results to details of the modelling procedure, have been made.

The Th II 401.913-nm line strength in the Solar Spectrum and the measured lifetime of the upper level of that transition combine to give a solar thorium abundance equal to its meteoritic abundance, within the estimated total error of 25%^{3–4}. In the solar spectrum, therefore, the line is unlikely to be contaminated by more than 20–30%. The neodymium line at 401.882 nm clearly lies in a complex region; comparison of its strength to that of the nearby low excitation lines of Nd II at 401.270 and 402.134 nm, in solar and laboratory spectra (refs 14, 23, 24; and

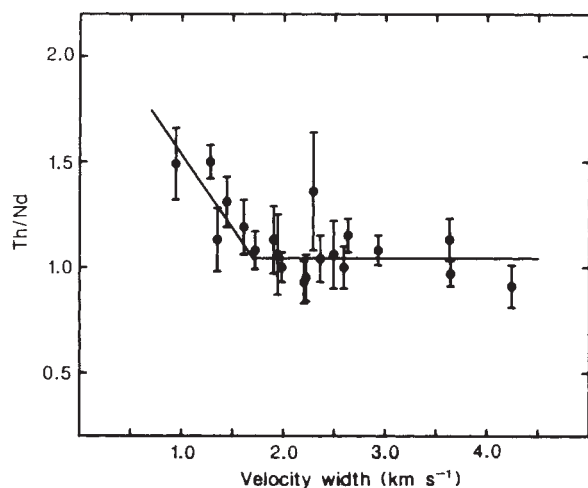


Fig. 2 Measured line strength ratios for all survey stars, as a function of net non-thermal velocity width (square root of sum of squares of micro-turbulent, macro-turbulent and rotational velocities in model fits). The final line strength ratios in column 6 of Table 3 are normalized and corrected for the upturn at small velocity widths by the following prescription:

$$\begin{aligned} \text{Th/Nd}_0 &= \text{Th/Nd}/1.043 \text{ for net width} > 1.70 \text{ km s}^{-1} \\ &= \text{Th/Nd}/(2.233 - 0.70 \times \text{width}) \\ &\text{for net width} < 1.70 \text{ km s}^{-1} \end{aligned}$$

for net width $< 1.70 \text{ km s}^{-1}$

Sceptical readers are invited to plot the raw (column 5, Table 3) ratios against temperature and against age to verify that this is the only systematic correlation present in the data. The presence of the effect shown here is not unexpected, given the simplified stellar atmospheric models used.

J. Blaise, personal communication), indicates that the feature measured could be contaminated by as much as 30% (but is also consistent with zero contamination).

Any blends with the programme lines which are not from low-lying levels of ionized metals will lead to variation of the Th/Nd line strength ratio with temperature and atmospheric electron pressure. A plot of the ratio against temperature for the programme stars shows no trend, and observations of the red giant stars HR1346 and 7869 (Tables 1 and 3) also reveal no unexpected behaviour. Any contamination present must therefore mimic the behaviour of the thorium line, suggesting perhaps a feature from another rare earth. HR5058, a peculiar red giant with very similar properties to HR7869^{25,26}, but with all elements synthesized in the s-process overabundant by large factors, has also been examined. Nd II 401.882 nm is enhanced by a factor of four in this star, as expected from this element's substantial s-process abundance^{8,9}. For thorium (with no s-process component), the line strength is 30% weaker than expected by comparison to HR7869; the origin of this discrepancy is believed to be either a slight difference in overall (non-s-process) heavy-element abundance levels, or a small fitting error due to the absence of any near-continuum points in the spectrum, magnified by the saturation of the line here. It is apparent that there is no contamination of the measured feature at 401.913 nm by rare earths with s-process abundance contributions.

Contamination by a mainly r-process species remains possible, although the only candidate discovered to date is Tb II 401.914 nm. Comparison of its laboratory line strength with the strengths of the four lines of Tb II seen in the solar spectrum^{14,23}, and with Th II 401.913 nm, points to a contamination of the thorium programme line of less than ~10% in the stellar spectra.

At least three details of the spectrum modelling procedures slightly affect the final abundance ratios. The nickel line position is evidently uncertain at the 0.0005-nm level. Tests with nickel line wavelengths slightly shifted from the value in Table 2 result in different Th line strengths; the variation of the Th/Nd ratio with temperature and other parameters, however, is (fortunately)

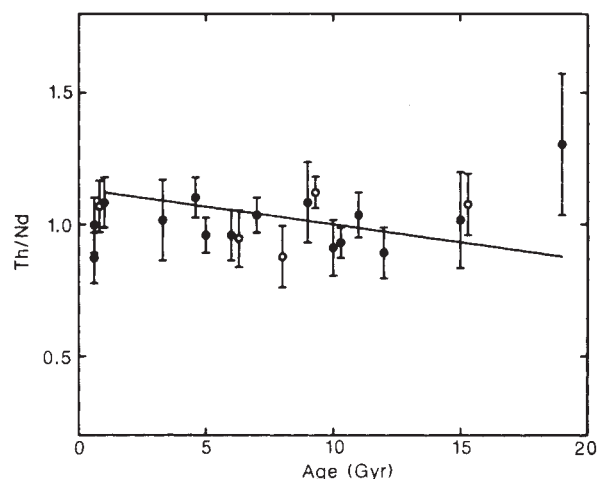


Fig. 3 Final normalized and corrected line strength ratios against stellar age. The solid line indicates the 2σ (σ is standard deviation) slope of a straight line fit to the increase of Th/Nd with time. The age scale assumed is the stellar evolutionary scale due to Vandenberg²⁹. Points plotted as open circles have net velocity widths less than 1.7 km s^{-1} in Fig. 2, and have been corrected for the velocity width effect seen there.

unaffected at the several per cent level. Similarly, errors in the assignment of a rotational velocity parameter have some effect on the final ratios; for $1.0 < v \sin i < 3.0 \text{ km s}^{-1}$, errors of 0.5 km s^{-1} in this parameter lead to errors in Th/Nd at the percent level. Model line-profile errors are also certainly present caused by the use of a simplified model atmosphere. The effects of these several errors are expected to be especially evident evident in spectra characterized by strong saturation of the iron/nickel blend. Figure 2 shows that such an effect is indeed present in the line ratio results. The Th/Nd ratio is seen to increase smoothly for the smallest line broadenings. The affected stars are neither all at one end of the temperature range of the sample, nor extremely young or extremely old. The raw ratios in Table 3 have therefore been corrected for this effect so that no trend with velocity width is apparent. The corrected and normalized (to a mean of 1.00) ratios are given in the final column of Table 3.

Th/Nd ratio versus age

The final abundance ratios are plotted in Fig. 3, with the corrected points shown as open circles. No evolution of the Th/Nd ratio with age is detectable. Even substantial errors in the age determinations of individual objects can have no influence on this conclusion. Although it remains possible that a fortuitous variation in r-process production ratios, or development of s-process abundances over time, could be conspiring to mask evolution in the Th/Nd ratio, it seems more likely that the ratio is in fact providing valid chronometric information. Assuming that to be the case, the following conclusions result.

No new information on the shape of $p(\tau)$ is provided by these data. They do, however, combine with the Solar System $^{232}\text{Th}/^{238}\text{U}$ chronometer to constrain the stellar-age scale. Consider two extreme models for the production function, $p(\tau)$.

Synthesis dominated by a single event before the formation of the sample stars always gives no variation of the Th/Nd ratio. But the Solar System data in this case provide a maximum age of 11.3 Gyr, with preferred values 2–3 Gyr younger^{27,28}. At the other extreme, synthesis at a constant rate throughout the life of the Galaxy leads via equation (1) to a fractional increase of Th/Nd with time of

$$(e^{\lambda_{\text{Th}} T} - 1)/\lambda_{\text{Th}} T > 1 + 0.025 \cdot T$$

A straight-line fit to the data in Fig. 3 yields a maximum increase (95% confidence level) of the observed Th/Nd ratio of

$0.0136(16/T_0)$ Gyr⁻¹. Assuming a 10% contamination of the stellar thorium measurements by Tb II 401.914 nm, these limits provide the constraint,

$$0.025 \leq 0.015(16/T_0)$$

or

$$T_0 \leq 9.6 \text{ Gyr}$$

Fits of exponential synthesis histories to Solar System data result in similarly short total timescales²⁸. In general, exponential production functions are also consistent with the stellar data if the derived total ages are less than 11 or 12 Gyr. Short timescales are therefore now indicated largely independently of uncertainties in the shape of $p(\tau)$ or in the chronometric production

ratios. Ages as great as 16–18 Gyr would only seem possible if some basic assumption of the analyses is incorrect.

It should be straightforward to improve on the observations and analysis reported here. It may not be so easy to discover how the theoretical stellar age scale might be some 30% too long, or how the idea of star-formation-linked element synthesis may be reconciled with the remarkable constancy of relative r- and s-process abundances over time in the Galaxy.

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- Schramm, D. N. *A. Rev. Astr. Astrophys.* **12**, 383–406 (1974).
- VandenBerg, D. A. *Astrophys. J. Suppl. Ser.* **51**, 29–66 (1983).
- Hauge, Ø. and Sørli, H. *Sol. Phys.* **30**, 301–308 (1973).
- Andersen, T. & Petkov, A. P. *Astr. Astrophys.* **45**, 237–238 (1975).
- Martin, W. C. *et al. J. phys. Chem. Ref. Data* **3**, 771–779 (1974).
- Moore, C. E. *A Multiplet Table of Astrophysical Interest* (NBS Tech. note no. 36, US Dept Commerce, Washington DC, 1959).
- Rutten, R. J. & van der Zalm, E. B. J. *Astr. Astrophys. Suppl.* **55**, 143–161 (1984).
- Seeger, P. A. *et al. Astrophys. J. Suppl. Ser.* **11**, 121–166 (1965).
- Allen, B. J. *et al. Adv. Nucl. Phys.* **4**, 205 (1971).
- Butcher, H. R. *Astrophys. J.* **199**, 710–717 (1975).
- Spite, M. & Spite, F. A. *Rev. Astr. Astrophys.* **23**, 225–238 (1985).
- Enard, D. in *Instrumentation in Astronomy IV, Proc. SPIE* **331**, 232–242 (1982).
- Middelberg, F. & Crane, P. in *Image Processing in Astronomy, Proc. 5th Coll. on Astrophysics* (eds Sedmak, E. *et al.*) 25–36 (Osservatorio di Trieste, 1979).
- Moore, C. E. *et al. The Solar Spectrum 2935A to 8770A* (NBS Monograph no. 61, US Dept Commerce, Washington DC, 1966).
- Blaise, J. *et al. Phys. Scr.* **29**, 119–131 (1984).
- Oertel, K. B. *Z. Physik* **236**, 90–96 (1970).
- Condon, E. & Shortley, G. *The Theory of Atomic Spectra* (Cambridge University Press, 1970).
- Mihalas, D. *Stellar Atmospheres* (Freeman, San Francisco, 1978).
- Gray, D. F. *The Observation and Analysis of Stellar Photospheres* (Wiley, New York, 1976).
- Warner, B. *Mon. Not. R. astr. Soc.* **136**, 381–388 (1967).
- Warner, B. *Observatory* **89**, 11–14 (1969).
- Fraser, R. & Suzuki, E. *Analyt. Chem.* **38**, 1770–1773 (1966).
- Meggers, W. F. *et al. Tables of Spectral-Line Intensities* (NBS Monograph no. 145, US Dept Commerce, Washington DC, 1975).
- King, A. S. *Astrophys. J.* **78**, 9 (1933).
- Warner, B. *Mon. Not. R. astr. Soc.* **129**, 263–297 (1965).
- Hoffleit, D. & Jaschek, C. *The Bright Star Catalogue 4th edn* (Yale University Observatory, New Haven, 1982).
- Meyer, B. S. & Schramm, D. N. in *Nucleosynthesis and its Implications on Nuclear and Particle Physics* (eds Audouze, J. & Mathieu, N.) 355–362 (Reidel, Dordrecht, 1986).
- Fowler, W. A. & Meisl, C. C. in *Cosmogonical Processes* (eds Arnett, W. D. *et al.*) 83–100 (VNU, Utrecht, 1986).
- VandenBerg, D. A. *Astrophys. J. Suppl. Ser.* **58**, 711–769 (1985).
- Duncan, D. K. *Astrophys. J.* **248**, 651–669 (1981).
- Soderblom, D. R. *Astrophys. J. Suppl. Ser.* **53**, 1–15 (1983).
- Soderblom, D. R. *Astr. J.* **90**, 2103–2115 (1985).
- Eggen, O. J. *Publs astr. Soc. Pacif.* **81**, 553–593 (1969).
- Eggen, O. J. *Publs astr. Soc. Pacif.* **83**, 251–279 (1971).
- Eggen, O. J. *Astrophys. J.* **182**, 821–837 (1973).
- Eggen, O. J. *Astr. J.* **92**, 910–951 (1986).
- Flannery, B. P. & Ayres, T. R. *Astrophys. J.* **221**, 175–185 (1978).
- Demarque, P. *et al. Astrophys. J.* **300**, 773–778 (1986).
- Patenaude, M. *Astr. Astrophys.* **66**, 235–239 (1978).
- Hirschfield *et al.* in *The HR Diagram IAU Symp. no. 80*, (eds Davis Philip, A. G. & Hayes, D. S.) 163 (Reidel, Dordrecht, 1987).
- Cayrel de Strobel, G. *et al. Astr. Astrophys. Suppl.* **59**, 145–186 (1985).
- Laird, J. B. *Astrophys. J. Suppl. Ser.* **57**, 389–404 (1985).
- Moore, C. E. *Atomic Energy Levels Vol. II* (NBS Circular No. 467, US Dept Commerce, Washington DC, 1952).
- Zalubas, R. & Corliss, C. H. *J. Res. NBS* **78A**, 163–246 (1974).

A common progenitor for neurons and glia persists in rat retina late in development

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Retrovirus-mediated gene transfer was used to mark cell lineages in vivo in the postnatal rat retina. Labelled clones contained up to three different cell types: three types of neurons or two types of neurons and a Müller glial cell. This indicates that a single retinal progenitor can generate remarkably diverse cell types near the end of development.

THE mammalian central nervous system (CNS) is extremely complex in both architecture and function. During the formation of many parts of the CNS, a variety of morphologically and functionally distinct cell types are generated simultaneously from common proliferative zones¹. Many fundamental aspects of this process are not understood, including the role of lineage in cell-type determination.

We² and others³ have recently developed a lineage-marking system based on retroviral vector-mediated gene transfer. Replication-incompetent retroviral vectors can stably introduce new genes into the genome of an infected cell. The integrated vector is inherited only by cells descended from the infected cell. Although retroviral vectors have been used previously to determine when cell lineages diverge^{4,5}, it has not been possible to identify single infected cells. We have used a vector which

expresses the β -galactosidase gene of *Escherichia coli* as a marker. The resulting β -galactosidase activity permits histochemical identification of individual cells containing the integrated vector. The ability to infect progenitor cells at various times during development and identify their descendants allows the determination of lineage relationships among different cell types. Most previous studies of vertebrate cell lineage have focused on early development^{4,6–8}, although lineages of neural crest derivatives⁹, and of glial cells in the optic nerve¹⁰ have been investigated later in development.

In this paper we report lineage relations among different cell types in the postnatal rat retina. The rodent retina is ideal for lineage studies: it is readily accessible to experimental manipulation, mitosis continues for about one week after birth^{11–15}, and the distinctive morphologies and layers of cells facilitate identification of the various cell types¹⁶. The functional diversity among retinal cells is representative of the cellular heterogeneity

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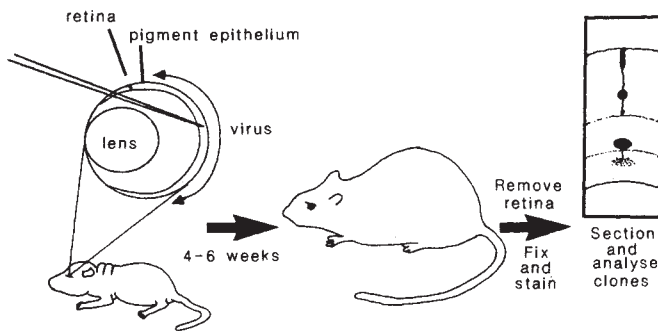


Fig. 1 Outline of experiments. Rat retinas were infected with the BAG retroviral vector², which expresses the *E. coli* β -galactosidase structural gene from the viral long terminal repeat promoter. Virus ($\sim 1 \mu\text{l}$) was injected into the back of the eye, between the pigment epithelium and the retina². Animals were killed at 4 to 6 weeks of age, and the infected retina was removed, fixed and stained for β -galactosidase activity with the chromogenic substrate X-gal as in ref. 2, except for the addition of 1 mM MgSO_4 to the staining reaction for some retinas (to intensify staining). Retinas were examined as whole mounts and then embedded and sectioned on a cryostat. Concentrated virus stocks were prepared as previously described². No wild-type helper virus was detected in virus stocks (not shown).

present in many parts of the CNS. Four different retinal cell types remain mitotic postnatally: rods, bipolar cells, amacrine cells and Müller glia^{11,13-15}. Rods are photoreceptor neurons, and bipolar cells are interneurons which connect radially the photoreceptor cells in the outer retina to cells in the inner retina. Amacrine cells are also interneurons, but with extensive lateral connections in the inner retina. Müller glial cells are non-neuronal cells which probably have metabolic and structural roles.

We have been able to mark numerous small clones in postnatal rat retinas and to label and identify all four mitotic cell types. We demonstrate here that rods, bipolar cells, and amacrine cells can have a common lineage until very late in development, and that rods, bipolar cells, and Müller glia also can share a lineage. In addition, rods and Müller glia, and possibly other retinal cells, can be derived from a common progenitor at its final mitosis, suggesting that cell type determination may occur at or after this final division.

Experimental design

The experiments are shown in the diagram in Fig. 1. Retinas were infected by injection of virus on the day of birth (P0), or on postnatal days P2, P4 or P7, and were removed and fixed four to six weeks later. Activity of β -galactosidase was detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) which forms a blue precipitate in infected cells^{2,3}. Clusters of labelled cells were clearly present in whole mount preparations² and in cross-sections of infected retinas (Figs 2a-f, 3a). Based on evidence presented later in this paper, we believe these clusters represent clones derived from individual infected progenitor cells.

Regions from several retinas were quantitatively analysed by examining serial sections and counting the number of cells of each type in each clone (Table 1). Regions to be analysed were chosen by selecting a series of sections in which the plane of section was such that each clone was contained in one or two sections. Identification of retinal cell types in sections was based on cellular morphology and on the location of cell nuclei within the retinal layers. Endothelial cells were labelled in the retinal capillaries of one P7 infection (Fig. 3b), and stained cells also were observed in the pigment epithelium of all infected eyes examined in this tissue (Fig. 3c). Retinal clones did not seem directly associated with either of these cell types, and no lineage

Fig. 2 (Opposite) X-gal-stained retinal sections showing clones of labelled cells derived from progenitors infected with the β -galactosidase vector. The retinal layers are labelled in a, c and d; rod inner and outer segments (os) are at the top in all sections. Rod cell nuclei are in the outer nuclear layer (onl), whereas the inner nuclear layer (inl) contains nuclei of the bipolar cells, Müller glia, and amacrine cells. The inner plexiform layer (ipl) contains processes from cells in the inl. a, Four rods (r) and a bipolar cell (bp). The labelled rod terminals are indicated (t), just above the bipolar cell. The labelled axon of the bipolar cell is visible in the inner plexiform layer. b, A rod and an amacrine cell (am). The X-gal label fills the entire rod, from the outer segment at the top of the section to the terminal below the rod soma. The dendrites of the amacrine cell are partially stained. c, Three rods and a Müller glial cell (mg) near the edge of the retina. The labelled main process of the Müller cell extends across the inner plexiform layer to the inner surface of the retina. A branch of this process ends on a blood vessel (arrowhead). The rod terminals are hidden by staining of the innermost rod soma. Nomarski interference contrast (d) and brightfield (e) views of a clone containing two adjacent rods, a bipolar cell and an amacrine cell. The dendrite of the bipolar cell is clearly visible, but the axon is only weakly stained. f, Five rods, a bipolar cell and a Müller glial cell. g, Camera lucida drawing of the clone shown in f. The rods and their terminals are shown in black, the bipolar cell and its dendrites in red, and the Müller cell and its processes in blue. The bipolar cell axon is not visible. All scale bars are 10 μm ; section thickness is 20 μm ; a, c, d and f taken with Nomarski optics.

analysis of these cells was attempted. Mock-infected retinas showed no background β -galactosidase activity with the staining conditions used².

Composition of clones

Labelled retinal cells were arranged in radial clusters in virtually every clone (Figs 2a-f, 3a). Most clones from P0 infections contained only a single cell type, but some clones did contain multiple cell types (Table 1). Rod and bipolar cell clones (Figs 2a and 3a) were the most common of these (18% of all P0 clones, including both P0 and P0(dil.) in Table 1), followed by rod and amacrine cell or rod and Müller glial cell clones (Fig. 2b and c; each about 3%). Three-cell-type clones were also observed, although less frequently: rod, bipolar cell, and amacrine (Fig. 2d; 0.6%) or rod, bipolar cell and Müller glial cell (Fig. 2e and f; 0.2%). The proportion of clones of each of the different cell-type combinations remained approximately constant in all P0 infections, with either undiluted or diluted virus (Table 1). They showed little change in infections at greater ages, except for a slight decrease in rod-amacrine cell clones. Overall, 74 different combinations of cell type and/or number of each type were observed among analysed clones (Table 1). No repeated pattern or ratio between different cell types is apparent. One-cell clones (isolated labelled cells) were the most common clones (36% at P0), and the percentage of these increased with age at infection.

Clone distributions and size

Clones from infections made before P7 were scattered over the entire retina, with some local variations in density. The

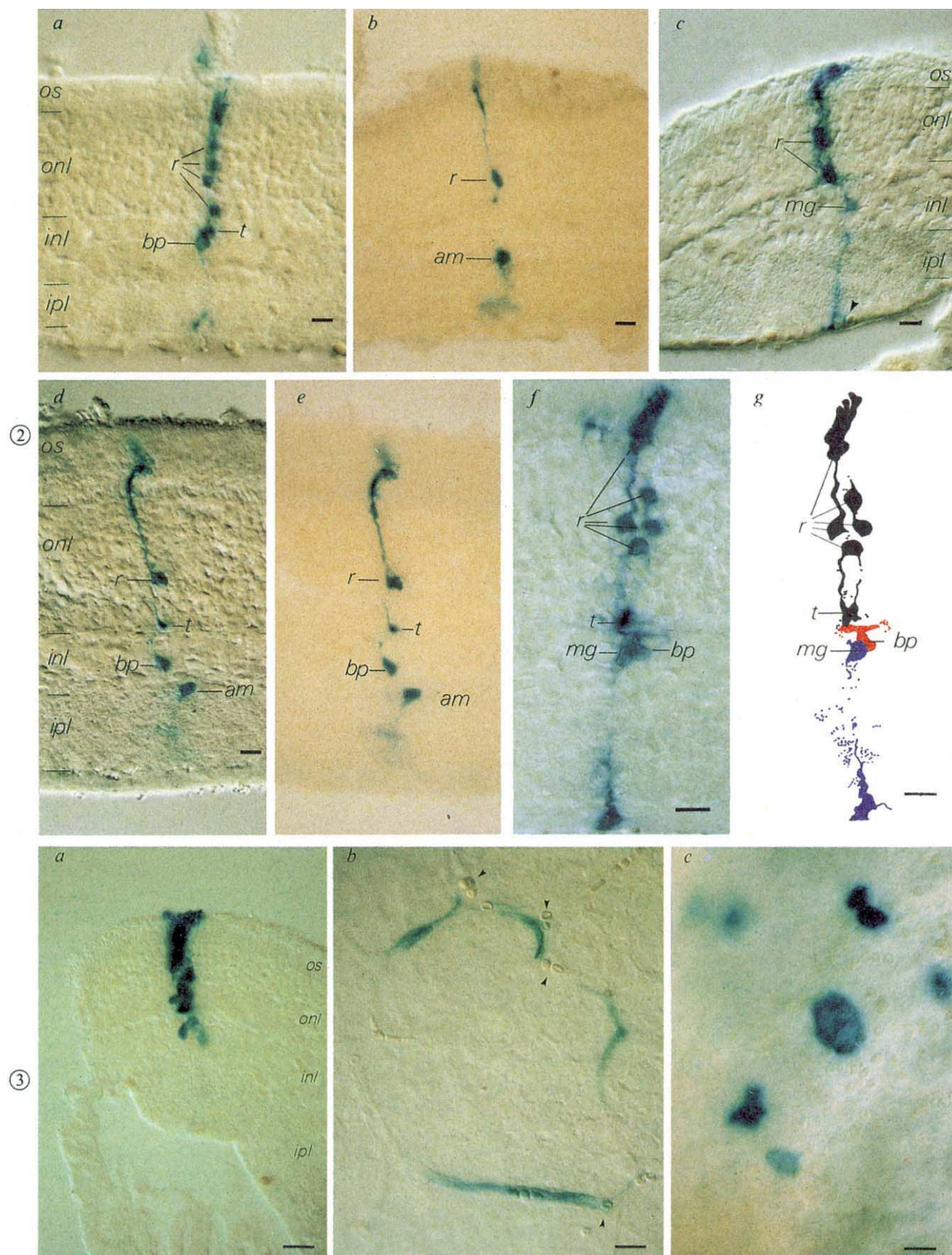


Fig. 3 Section from a P0-infected retina showing a large clone near the edge of the retina: 18 labelled rods are present in the outer nuclear layer (onl) and 4 labelled bipolar cells are present in the inner nuclear layer (inl). The labelled cells are in several planes of focus. Other abbreviations as Fig. 2. *b*, The inner surface of a retinal whole mount from a P7 infection. Several elongated endothelial cells are labelled in the retinal capillaries. Red blood cells are visible in the capillaries (arrowheads, examples). *c*, Whole mount showing labelled cells in the pigment epithelium from a P0 infection. Scale bars, 20 μ m.

Table 1 Combinations of cell number and type present in labelled clones

Cell types in clones	Cell numbers in clones	Number (per cent) of clones from infection on postnatal day				
		P0	P0(dil.)	P2	P4	P7
Rod only	1r	325(32.0%)	205(44.7%)	200(57.6%)	543(58.8%)	72(69.2%)
	2r	141(13.9%)	97(21.1%)	59(17%)	146(15.8%)	11(10.8%)
	3r	120(11.8%)	34(7.4%)	18(5.2%)	19(2.1%)	
	4r	65(6.4%)	20(4.4%)	7(2.0%)	4(0.4%)	
	5r	6(0.6%)	2(0.4%)			
	6r	6(0.6%)	2(0.4%)			
	7r		1(0.2%)			
	8r	2(0.2%)	1(0.2%)			
	10r	2(0.2%)				
	11r		1(0.2%)			
	12r		1(0.2%)			
	13r		1(0.2%)			
	Total	667(65.6%)	365(79.5%)	284(81.8%)	712(77.1%)	83(79.8%)
	1b	5(0.5%)	2(0.4%)	2(0.6%)	33(3.6%)	
Bipolar only	2b			1(0.3%)	3(0.3%)	
Amacrine only	1a	29(2.9%)	9(2.0%)	2(0.6%)	7(0.8%)	
Müller glia only	2a		1(0.2%)			
Rod + bipolar	1m			1(0.3%)	11(1.2%)	11(10.6%)
	1r + 1b	25(2.5%)	8(1.7%)	19(5.5%)	69(7.5%)	
	2r + 1b	50(4.9%)	19(4.1%)	6(1.7%)	19(2.1%)	
	1r + 2b	2(0.2%)	1(0.2%)	1(0.3%)		
	2r + 2b	1(0.1%)	3(0.7%)		2(0.2%)	
	3r + 1b	62(6.1%)	18(3.9%)	2(0.6%)	2(0.2%)	
	4r + 1b	36(3.5%)	8(1.7%)	1(0.3%)	1(0.1%)	
	3r + 2b	2(0.2%)		1(0.3%)		
	4r + 2b	3(0.3%)				
	5r + 1b	12(1.2%)		1(0.3%)		
	6r + 1b	6(0.6%)				
	5r + 2b	2(0.2%)		1(0.3%)		
	7r + 1b	3(0.3%)	1(0.2%)			
	6r + 2b	2(0.2%)				
	8r + 1b	1(0.1%)				
	9r + 1b	1(0.1%)				
	10r + 1b	2(0.2%)				
	11r + 1b		1(0.2%)			
	12r + 3b	1(0.1%)				
	18r + 4b	1(0.1%)				
	Total	212(20.8%)	59(12.8%)	31(8.9%)	93(10.1%)	
Rod + amacrine	1r + 1a	13(1.3%)	5(1.1%)	1(0.3%)	5(0.5%)	
	1r + 2a	1(0.1%)	1(0.2%)			
	2r + 1a	3(0.3%)	2(0.4%)		2(0.2%)	
	3r + 1a	8(0.8%)	1(0.2%)			
	2r + 2a				1(0.1%)	
	4r + 1a	6(0.6%)				
	3r + 2a	1(0.1%)				
	5r + 1a	2(0.2%)				
	Total	34(3.3%)	9(1.9%)	1(0.3%)	8(0.9%)	
	1r + 1m	2(0.2%)	1(0.2%)	7(2.0%)	19(2.1%)	2(1.9%)
Rod + Müller glia	2r + 1m	7(0.7%)	3(0.7%)	1(0.3%)	7(0.8%)	
	3r + 1m	6(0.6%)	2(0.4%)	3(0.9%)		
	4r + 1m	9(0.9%)	1(0.2%)	1(0.3%)		
	5r + 1m	4(0.4%)	2(0.4%)	1(0.3%)		
	6r + 1m	2(0.2%)				
	7r + 1m	1(0.1%)				
	10r + 1m	1(0.1%)				
	12r + 1m	1(0.1%)				
	Total	33(3.2%)	9(1.9%)	13(3.7%)	26(2.8%)	2(1.9%)
	1r + 1b + 1a	1(0.1%)	1(0.2%)		1(0.1%)	
Rod + bipolar + amacrine	2r + 1b + 1a	1(0.1%)				
	4r + 1b + 1a	1(0.1%)				
	5r + 1b + 1a	2(0.2%)				
	6r + 1b + 1a	1(0.1%)				
	7r + 1b + 1a	1(0.1%)				
	8r + 1b + 1a	1(0.1%)				
	Total	8(0.8%)	1(0.2%)		1(0.1%)	
Rod + bipolar + Müller glia	2r + 1b + 1m	1(0.1%)				
	5r + 1b + 1m	1(0.1%)				
	12r + 1b + 1m	1(0.1%)				
INL	1i	8(0.8%)	2(0.4%)	8(2.3%)	21(2.3%)	7(6.7%)
	2i				1(0.1%)	
Rod + INL	1r + 1i	5(0.5%)	1(0.2%)	2(0.6%)	3(0.3%)	1(1.0%)
	2r + 1i	1(0.1%)	1(0.2%)	1(0.3%)	1(0.1%)	
	3r + 1i	7(0.7%)			1(0.1%)	
	4r + 1i	1(0.1%)				
	5r + 1i	1(0.1%)				
	4r + 3i				1(0.1%)	
	Total	15(1.5%)	2(0.4%)	3(0.9%)	6(0.7%)	1(1.0%)
Rod + Müller glia + INL	3r + 1m + 1i	1(0.1%)				
Müller glia + INL	9r + 1m + 1i	1(0.1%)				
	1m + 1i				1(0.1%)	
Total clones scored		1,016(100%)	459(100%)	347(100%)	923(100%)	104(100%)

All combinations of cell numbers and types observed in quantitatively analysed retinas are shown at different ages of infection. Clones are grouped by the cell types they contain (for example, rod + bipolar), and listed from smallest to largest in each group. Cell types are: r, rod; b, bipolar; a, amacrine; INL or i, labelled cell of undetermined type in the inner nuclear layer; m, Müller glial cell. Totals are provided for all groups containing more than three combinations, and for scored clones at each age. Retinas were infected with $\sim 1 \mu\text{l}$ of a 8×10^7 CFU ml^{-1} virus stock, except for the P0(dil.) retinas which were infected with $\sim 1 \mu\text{l}$ of 1:25 or 1:30 dilutions of this stock. Data at most ages is pooled from several retinas; P0:3 retinas (2 contained $\sim 5,000$ total clones, the other ~ 860), P0(dil.): 3 retinas (~ 400 clones each), P2:1 retina (~ 800 clones), P4:2 retinas (~ 350 and ~ 600 clones), P7:2 retinas (46 and 56 clones).

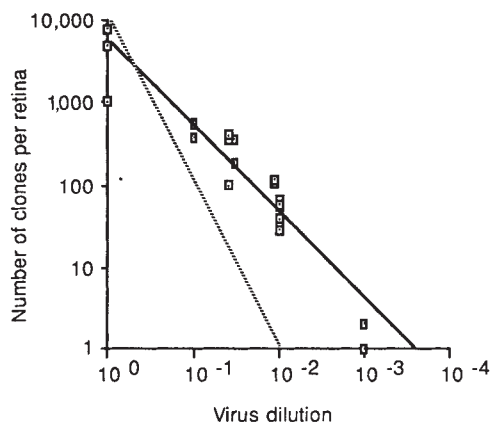


Fig. 4 Number of clones per retina plotted against various dilutions of injected virus. Solid line, linear regression for the plotted points with a slope of -1.1 . Clones generated by independent infection events should decrease linearly with dilution (a slope of -1). If all clones were generated by two independent infection events, the number of clones would decrease as the square of the dilution (a slope of -2), shown for comparison by the dashed line.

separation between clones in the most densely infected retinas analysed varied from 10 to several hundred cell diameters in different regions of each retina. Infections on P7 resulted in labelled clones only near the edge of the retina. The average clone size was largest at P0 (2.5 cells per clone), and decreased in later infections to an average of 1.1 at P7, with intermediate values of 1.6 and 1.4 at P2 and P4, respectively. Clones in the peripheral retina tended to be larger than those in the central retina at all ages, with largest clones invariably near the edge of the retina (an example is shown in Fig. 3a). The number of cells per clone ranged from 1 to 22 in retinas infected at P0, from 1 to 7 in retinas infected at P2 and P4, and from 1 to 2 in P7 infections (Table 1).

Number of clones per retina

Retinas infected on P0 with $\sim 1 \mu\text{l}$ of a concentrated virus stock (8×10^7 G418-resistant colony forming units (CFU) per ml, assayed on NIH 3T3 cells) contained several thousand clones. The most densely infected retinas were estimated to have 5,000–10,000 clones. Assuming half of the virus was absorbed by the pigment epithelium, this is an efficiency of about 25% relative to NIH 3T3 cells. Since viral integration and β -galactosidase expression probably require mitosis (see discussion), and only 50–60% of the retinal progenitor cells were mitotic at birth^{12,13}, the actual efficiency may be twofold higher. The number of clones per retina decreased with age at infection: an ~ 10 -fold decrease between P0 and P4, and an additional 10-fold decrease between P4 and P7.

Virus dilutions

To demonstrate that the number of clones was dependent on the amount of virus injected, $\sim 1 \mu\text{l}$ of each of several dilutions of the virus stock were injected. In all cases, the number of clones decreased linearly with virus dilution, demonstrating that each clone was indeed the result of a single infection event (Fig. 4). Scatter in these data is probably due to variability in the volumes of virus injected or to leakage of virus from the injection site. The separation between clones increased in infections with dilute virus. The range of average clone sizes in six P0 infections with undiluted and diluted virus were similar (2.3, 2.4 and 3.0 compared to 2.0, 2.1 and 2.2 cells per clone, respectively).

Discussion

Clones which contained multiple cell types (Table 1 and Figs 2 and 3) indicate that two or three retinal cell types can arise

from a common lineage near the end of cell proliferation. In some cases, a single progenitor can generate rods, a bipolar cell and an amacrine cell; or rods, a bipolar cell and a Müller glial cell. In addition, a single retinal precursor can produce two cells of different types at the last mitosis. Many clones which contained only two cells, of different types, were observed (Fig. 2b). It seems likely that many of these clones were the product of a terminal mitosis, although death of the other cells in an initially larger clone remains a possibility. In infections at P7, when most mitoses are the final ones, a few rod and Müller glial cell clones were observed. In this case, it seems certain that some of these progenitors were completing their final division.

The approximately linear decrease in the number of labelled clusters with virus dilution, as well as the invariance of cluster size and cell type composition with dilution, indicate that the clusters were clones, derived from the infection of single progenitor cells. The possibility that labelled clusters were generated by aggregates of infectious virus is unlikely. Analysis of proviral copy number by probing Southern blots of DNA from clones generated by infection of mouse fibroblasts *in vitro* demonstrated that infection always resulted in the integration of a single provirus (unpublished observations). Because $\sim 50\%$ of the virus was recovered after concentration, and because retinal infection was very efficient, large clumps of infectious virus that generated the complex clusters could not have occurred. Moreover, the ratio of defective particles to infectious particles for retroviruses is high, between 100 and 1,000 to one (unpublished observations and S. Goff, personal communication). Infection of two independent progenitors by a clump of 200–2,000 particles which disintegrates at the time and site of infection is very unlikely. Labelling of multiple cells by diffusion of either β -galactosidase or reacted X-gal substrate through intercellular junctions or across synapses did not seem to occur. Rods are connected to each other by gap junctions¹⁷, and all the labelled neuronal cell types have multiple synapses, yet the most common clones were single labelled cells. These single-cell clones may have been generated by integration of the virus into the genome of only one daughter cell during the final mitosis of a progenitor cell, or by the loss of labelled sibling cells due to cell death^{18,19}. It is unlikely these cells were postmitotic at the time of infection because previous studies have demonstrated that retroviral integration requires an S-phase²⁰. The absence of clones in the postmitotic central retina in P7 infections, and the lack of labelled cones, horizontal cells, or ganglion cells (all of which cease division before birth^{11,13,14}), indicates that successful infection in the retina probably also requires an S-phase.

The labelling of all four retinal cell types generated postnatally, in proportions similar to those determined for postnatal mouse retina¹³, suggests that neither infection nor β -galactosidase gene expression were selective or harmful to the different cell types. The high infection efficiency also argues against a deleterious effect of the introduced gene. Although some variations in staining intensity existed, the readily detectable staining observed in retinal cells, as well as pigment epithelial cells and endothelial cells, indicates relatively high levels of β -galactosidase gene expression. Expression of other genes in retroviral vectors in progenitor cells and *in vivo* has varied^{21–26}.

The labelled clones showed a radial orientation in the retina. The lack of mixing of labelled and unlabelled cells implies that little lateral migration occurred. This is consistent with previous observations of retinal development using tritiated thymidine autoradiography^{11–15}, which showed that cells migrate radially inward after their final mitosis. The findings of the small number of cells in most clones and that the largest clones are near the edge of the retina are also in agreement with these studies.

We think the most plausible explanation for the multitude of clone types is the existence of a common progenitor for all four cell types. An alternative is the presence of multiple progenitors

which produce restricted combinations of one to three cell types. Clones labelled by infections earlier in development may determine if a single clone can contain all cell types. Preliminary results from the infection of embryonic chick retinas indicate that large clones usually contain many cells in both the inner and outer nuclear layers (C.L.C., unpublished data). This is consistent with a single-precursor model, as are some observations of human retinoblastoma cell lines in culture²⁷⁻²⁹. Retinal cell type is probably not determined by invariant lineages, as in the nematode, *Caenorhabditis elegans*³⁰, considering the large number of cell type combinations and ratios within clones. The possibility of an invariant lineage obscured by cell death is unlikely, because estimates of cell death for the various labelled cell types are 3% to 8% (ref. 19). A model in which cell fate is independent of lineage and is instead determined by environmental factors, as in the development of the *Drosophila* eye^{31,32}, seems more likely. Recent examination^{33,34} of a *Drosophila* mutant, sevenless, where a specific photoreceptor cell undergoes a homeotic switch to become a non-neuronal cell, may provide clues for determination in the vertebrate retina as well. Retinal regeneration³⁵ and transdifferentiation³⁶ studies in the chick

suggest environmental interactions can influence cell fates in a vertebrate eye.

The lineage relationship between neurons and glia in the CNS is an unresolved issue^{1,37}. Although both cell types initially form in the same proliferative zones, it is not clear whether they derive from the same precursor cells throughout development, or from lineages which diverge at some time before the end of neurogenesis. The results presented here show that retinal neurons and glia can share a common lineage, probably throughout development. Development in other parts of the CNS resembles retinal development¹. It will be interesting to see if multiple cell type clones occur elsewhere in the CNS and whether radial lineages may contribute to the assembly of functional architectures, such as the orientation and ocular dominance columns³⁸ of the visual cortex.

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- Jacobson, M. *Developmental Neurobiology* 2nd edn, 27-55 (Plenum, New York, 1982).
- Price, J., Turner, D. & Cepko, C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 154-160 (1987).
- Sanes, J. R., Rubenstein, J. L. R. & Nicolas, J. *EMBO J.* **5**, 3133-3142 (1986).
- Soriano, P. & Jaenisch, R. *Cell* **46**, 19-29 (1986).
- Lemischka, I. R., Ruet, D. H. & Mulligan, R. C. *Cell* **45**, 917-927 (1986).
- Kimmel, C. B. & Warga, R. M. *Science* **231**, 365-368 (1986).
- Mintz, B. & Sanyal, S. *Genetics* **64**, suppl. 43-44 (1970).
- Moody, S. A. & Jacobson, M. J. *Neurosci.* **3**, 1670-1682 (1983).
- Le Douarin, N. M. *Science* **231**, 1515-1522 (1986).
- Raff, M. C., Miller, R. H. & Noble, M. *Nature* **303**, 390-396 (1983).
- Sidman, R. L. *The Structure of the Eye* 487-506 (Academic, New York, 1961).
- Denham, S. J. *Embryol. exp. Morph.* **18**, 53-66 (1966).
- Young, R. W. *Anat. Rec.* **212**, 199-205 (1985).
- Carter Dawson, L. D. & LaVail, M. M. *J. comp. Neurol.* **188**, 263-272 (1979).
- Blanks, J. C. & Bok, D. J. *comp. Neurol.* **174**, 317-328 (1977).
- Rodieck, R. W. *The Vertebrate Retina* 338-368 (Freeman, San Francisco, 1973).
- Raviola, E. & Gilula, N. B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1677-1681 (1973).
- Lam, K., Sefton, A. J. & Bennett, M. R. *Dev. Brain Res.* **3**, 487-491 (1982).
- Young, R. W. *J. comp. Neurol.* **229**, 362-373 (1984).
- Varmus, H. E., Padgett, T., Haesley, S., Simon, G. & Bishop, J. M. *Cell* **11**, 307-319 (1977).
- Keller, G., Paige, C., Gilboa, E. & Wagner, E. F. *Nature* **318**, 149-154 (1985).
- Stewart, C. L., Vanek, M. & Wagner, E. F. *EMBO J.* **4**, 3701-3709 (1985).
- Eglitis, M. A., Kantoff, P., Gilboa, E. & Anderson, W. F. *Science* **230**, 1395-1398 (1985).
- Huszar, D. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8587-8591 (1985).
- Williams, D. A., Orkin, S. H. & Mulligan, R. C. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2566-2570 (1986).
- Robertson, E., Bradley, A. & Kuehn, M. *Nature* **323**, 445-448 (1986).
- Kyritsis, A., Tsokos, M., Triche, T. & Chader, G. *Nature* **307**, 471-473 (1984).
- Jiang, Q., Lim, R. & Blodi, F. C. *Expl. Eye Res.* **39**, 207-215 (1984).
- Canton, M. D., Hinton, D., Gray, D., Phillips, R. A. & Gallie, B. B. L. *Invest. Ophthalmol. Vis. Sci.* **25**, suppl. 259 (1985).
- Sulston, J. E. & Horvitz, H. R. *Dev. Biol.* **56**, 110-156 (1977).
- Ready, D. F., Hanson, T. E. & Benzer, S. *Dev. Biol.* **53**, 217-240 (1976).
- Tomlinson, A. & Ready, D. F. *Dev. Biol.* **120**, 366-374 (1987).
- Hafen, E., Basler, K., Edstroem, J. & Rubin, G. M. *Science* **236**, 55-63 (1987).
- Banerjee, U., Renfranz, P., Pollack, J. & Benzer, S. *Cell* **49**, 281-291 (1987).
- Coulombre, J. L. & Coulombre, A. J. *Dev. Biol.* **12**, 79-92 (1965).
- Moscona, A. A., Brown, M., Degenstein, L., Fox, L. & Soh, B. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 7239-7243 (1983).
- Levitt, P., Cooper, M. L. & Rakic, P. *J. Neurosci.* **1**, 27-39 (1981).
- Hubel, D. H. & Wiesel, T. N. *Proc. R. Soc. B* **198**, 1-59 (1977).

LETTERS TO NATURE

Possible explanation of the γ -ray light curve and time variability in Cygnus X-3

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Very high-energy (VHE) and ultra high-energy (UHE) γ rays, at around 1 TeV and 1 PeV respectively, have been observed from Cygnus X-3 predominantly in two distinct regions of orbital phase. Here we investigate whether this can be understood in terms of the accretion disk corona model of White and Holt¹ developed to explain the asymmetric light curve of the low-mass X-ray binary system X1822-371. In this model, two bulges in the outer rim of the accretion disk are required to fit the observed X-ray light curve and we find these could provide target material for the production of the γ rays observed at two distinct phases. We show also that magnetic steering effects may be inevitable in Cyg X-3 and may lead to a natural explanation to the observed variability in flux and phase of γ -ray emission.

The phases at which γ -ray emission has been observed from Cyg X-3 (see, for example, the reviews in refs 2-4), $\phi \sim 0.25$

and $\phi \sim 0.63$, are not easily accounted for in models⁵⁻⁸ where high energy particles accelerated in the vicinity of the neutron star interact in the atmosphere of its stellar companion. A model⁹ in which the target is in the form of a fountain of stellar material ejected from the companion disrupted by a high-energy proton beam has been suggested and is able to give emission at two phases close to those required. In another model¹⁰, a wake in a stellar wind accretion flow may be responsible for the emission at $\phi \sim 0.63$. We consider yet another possibility, that bulges in an accretion disk may form the target material. This possibility is particularly appealing as bulges in the outer rim of the accretion disk are required in the accretion disk corona (ADC) model of White and Holt¹. This model has had considerable success in fitting the optical, infrared and ultraviolet light curves¹¹ as well as the X-ray light curve of X1822-371, and has been applied to the X-ray light curve of Cyg X-3¹.

In the ADC model¹, X rays produced near the neutron star are scattered in a high-temperature low-density plasma, the ADC, which may result from evaporation off the accretion disk due to X-ray heating¹². The ADC forms an extended X-ray source which is partly occulted by the stellar companion and the accretion disk. To give the observed X-ray light curve two bulges must be present in the outer rim of the disk, the main bulge at the confluence of the accretion stream with the disk, and one upstream of this referred to as the 'pre-bulge'. We would expect the amount of matter encountered per unit area on traversing the bulges, the 'grammage', to be of the same order

Table 1 Possible dimensions of Cyg X-3 system

	Stellar mass ($M_{\odot}$)	Stellar radius ($R_{\odot}$)	Binary separation ($R_{\odot}$)	Disk radius* ($R_{\odot}$)	Orbital inclination† (deg.)
Main-sequence dwarf	0.55	0.54	1.80	0.8	66
Helium star	3.85	1.18	2.49	0.7	60

* Disk assumed to fill Roche lobe around neutron star.

† Values assumed in present work.

of magnitude as that across the accretion disk. For a proton luminosity of $\sim 10^{39}$ erg s $^{-1}$ (see, for example, ref. 13) we require $\dot{M} > 5 \times 10^{18}$ g s $^{-1}$ assuming the conversion of kinetic energy of infalling matter to relativistic particles takes place near the neutron star's surface and we estimate the grammage using the standard thin accretion disk model² to be in excess of ~ 200 g cm $^{-2}$ at a radius of $0.8 R_{\odot}$ (see below). Clearly, there is likely to be enough material for the production of γ rays via π^0 decay and so it is feasible that bulges in the accretion disk may form the target material.

If we know the location of possible target material within the binary system we may estimate at which orbital phases we would expect γ -ray emission to occur assuming that protons are accelerated and emitted isotropically from a region close to the neutron star. The best fitting bulge profiles are given in Table 1 of White and Holt¹ and the peak bulge height is given in Fig. 10 of ref. 1 as a function of orbital inclination for various accretion disk radii. The inclination and likely disk radius depends on the nature of the companion star. The stellar companion is likely to be either a main-sequence dwarf or a helium star which has evolved in the binary system¹⁴. For a 4.8 h orbital period and assuming that the compact object is a neutron star of mass $1.4 M_{\odot}$, its companion fills its Roche lobe, and that the accretion disk probably fills the Roche lobe around the neutron star¹⁵ we obtain the masses and dimensions given in Table 1. We also give in the table the values of orbital inclination we adopt which are close to those which would require the smallest

bulges to fit the X-ray data. Our conclusions below are unaffected by this choice for reasonable values of the inclination.

We are now in a position to work out the expected phases of γ -ray emission, that is, those phases during which our line of sight to the neutron star is intersected by one or other of the bulges. For both possible companions, assuming rectilinear propagation of protons accelerated near the neutron star, we find we would expect a burst of γ -ray emission at phase $\phi = 0.85$ – 0.99 due to interactions in the main bulge and a burst at $\phi = 0.24$ – 0.29 due to interactions in the pre-bulge. Since Cyg X-3 does not undergo an eclipse the phase origin used needs some clarification. Here we use the X-ray phase, that is, the phase origin is essentially coincident with the X-ray minimum in the early X-ray data, and this is used when analysing γ -ray observations. In ref. 1 phases are arbitrarily given with respect to the line joining the centres of the stars and we have estimated the X-ray phases given above by comparing the fitted X-ray light curve from Fig. 9 of ref. 1 with that of Fig. 1a of van der Klis and Bonnet-Bidaud¹⁶ (note that the eclipse centre would be at X-ray phase $\phi \approx 0.1$ if the inclination were greater).

The γ -ray emission observed at $\phi \sim 0.25$ could be due to the pre-bulge. The fits of White and Holt¹ are based on early data and since then the light curve has undergone a fairly dramatic change in shape¹⁶ with apparently more occultation of the ADC by bulges taking place at $\phi \sim 0.5$ – 0.8 than in the earlier X-ray data. Unfortunately no ADC model fits have been performed on these more recent X-ray data; however, we would point out that the phases of γ -ray emission by the main bulge would be closer to $\phi \sim 0.63$ than those we obtained above. More detailed predictions must await ADC model fits to the more recent data.

For both possible stellar companions mentioned above steering of the proton beam by the magnetic field of the companion star might occur. Such magnetic steering has been proposed by Gorham and Learned¹⁷ recently to explain the observation of VHE γ rays from Hercules X-1 during an X-ray eclipse¹⁸. While the nature of the stellar companion remains unknown, it is impossible to make any reliable predictions of such effects in Cyg X-3. But if the companion star is a main-sequence dwarf of about half a solar mass and half a solar radius, it is likely to be of spectral class¹⁹ between K5 and M2. Recently, it has become possible to measure the surface magnetic fields of G and K dwarfs and the latter are found²⁰ typically to have 20–80% of their surface area covered with magnetic fields in the range 500–3,000 G. The fields of these stars are not well represented by single dipoles, but rather by two or three dipoles embedded below the star's surface.

Nothing is known about the magnetic fields of helium stars. Such stars are considerably hotter than main-sequence hydrogen-burning stars. The hottest stars for which information about magnetic fields are known are Ap stars with strong helium lines for which magnetic fields are typically well over a kilogauss²¹. In Ap stars the dipole axis appears to be randomly orientated with respect to the rotation axis and rotates with the star. For such a situation, and also for the case of the main sequence dwarf companion, the field configuration in the frame rotating with the binary system would not vary with the orbital motion if, as seems likely, co-rotation takes place.

In the binary system, the field will be complicated by the presence of the accretion disk which may amplify it and by the

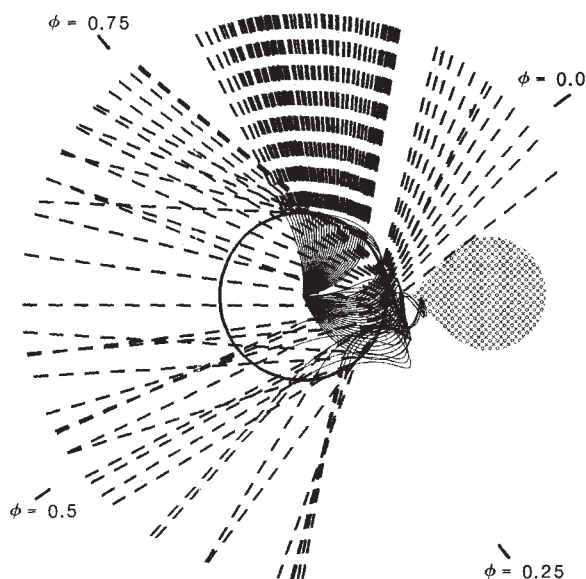


Fig. 1 Particle and γ -ray trajectories as viewed looking down the orbital axis. The accretion disk (heavy circle) is assumed to lie in the orbital plane and the dimensions of the system used are those given in Table 1 for a main-sequence dwarf companion (shown hatched). Trajectories of protons that interact to produce a γ ray at an angle to the orbital axis which is close to the binary inclination are plotted (solid lines) together with the directions of γ rays which result (dashed lines). Orbital phases, ϕ , appropriate to Cyg X-3 are indicated (see text).

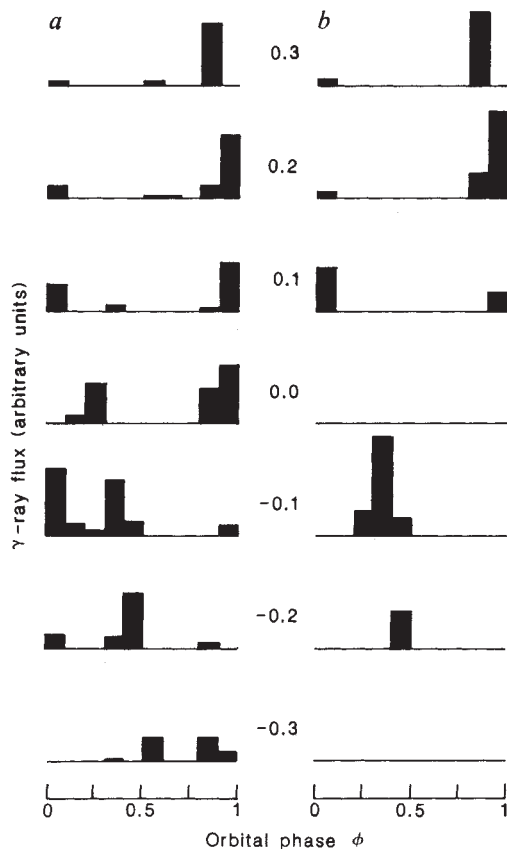


Fig. 2 The γ -ray light curves for various ratios of magnetic moment to rigidity (numbers attached to the curves in units of $R_{\odot}^3 \text{ G TV}^{-1}$) for *a*, isotropic emission and *b*, emission at an angle $\alpha \sim 15^\circ$ above the orbital plane.

neutron star's field which may have a poloidal component as high as $\sim 10^3 \text{ G}$ at the radii of interest²². Nevertheless, an idea of the importance of the magnetic steering effects expected can be obtained by modelling the field in the binary system as just that of a single dipole located at the centre of the stellar companion and orientated parallel to the orbital axis. For this reason we follow, in three dimensions, the trajectories in such a magnetic field of protons produced near the neutron star until they either interact with the accretion disk, strike the star, or escape from the binary system. This has been done for: (1) isotropic emission of energetic particles; and (2) emission of particles in directions at some angle α above the orbital plane, their directions forming a cone of half angle $(90^\circ - \alpha)$ about the orbital axis. The latter case may apply if acceleration takes place along the magnetic axis of a neutron star which is not aligned with its spin axis. Such a model might avoid the problems associated with neutrino heating of the stellar companion²³ and may allow modulation of the VHE γ -ray flux with spin period of the neutron star as has probably been observed²⁴. In all that follows, we assume that the accelerator produces a monoenergetic particle beam or a very flat ($\sim E^{-2}$ differential) spectrum. For such a particle spectrum, Hillas⁹ has shown that a cascade in target material will lead naturally to the observed γ -ray spectrum of Cyg X-3. For a monoenergetic beam the magnetic steering would allow the γ -ray light curve to peak at the same phases at all energies.

A typical result is shown in Fig. 1 which shows particle and γ -ray trajectories as viewed looking down the orbital axis for a ratio of stellar magnetic moment to particle rigidity of $0.2 R_{\odot}^3 \text{ G TV}^{-1}$. Trajectories have been followed for values of the ratio of magnetic moment to rigidity ranging from -0.3 to $0.3 R_{\odot}^3 \text{ G TV}^{-1}$.

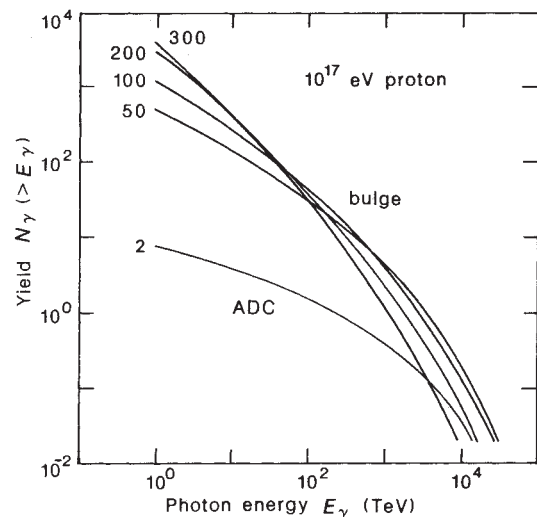


Fig. 3 The average γ -ray flux produced by a 10^{17} eV proton in 2 g cm^{-2} of hydrogen (ADC) compared to that produced in a cascade in the bulges of density $10^{-7} \text{ g cm}^{-3}$ for various grammages (numbers attached to the curves give the grammage in g cm^{-2}).

TV^{-1} and the resulting γ -ray light curves are plotted in Fig. 2*a* for isotropic particle emission and in Fig. 2*b* for the case of energetic particles emitted at an angle $\alpha \sim 15^\circ$ above the orbital plane.

Even though the magnetic field configuration in Cyg X-3 is quite unknown, these results show that for the kilogauss magnetic fields we might expect to be present in the Cyg X-3 system magnetic steering of beams of particles of energies up to and exceeding $\sim 1 \text{ PeV}$ may play an important part in determining the VHE and UHE γ -ray light curve. Furthermore, in the case of main-sequence dwarf stars of spectral class late K to early K, time variability has been observed on relatively short time-scales (5–20 yr) similar to that observed for the magnetic field of the Sun²⁵. This could lead, for example, to a change from emission occurring predominantly at $\phi \sim 0.25$ to emission occurring mainly at $\phi \sim 0.63$ as appears to have occurred between 1983 and 1984 while observations of this source were made at Haverah Park²⁶. Inspection of Fig. 2*b* shows that such magnetic field variations could give a natural explanation also for the time variability of γ -ray flux which had apparently been observed^{3,27}.

We now consider interactions of protons in the ADC which will give rise to a d.c. component of γ rays. The flux from the ADC relative to that from the bulges must not be inconsistent with the air shower data. In Fig. 3 we show the average γ -ray flux produced by a 10^{17} eV proton in 2 g cm^{-2} of hydrogen (our estimate of the grammage through the ADC). This is compared with that produced in a cascade in the bulges for a density of $10^{-7} \text{ g cm}^{-3}$ (assuming it to be similar to that in the disk) and grammages in the bulges ranging from 50 to 300 g cm^{-2} . Based on the Haverah Park data²⁸ above 1 PeV and Kiel data²⁹ above 2 PeV we obtain 95% upper limits³⁰ to the ratio of d.c. flux to excess flux during phases of γ -ray emission of 0.09 ± 0.01 and 0.13 ± 0.03 respectively. From our result in Fig. 3, we find that the predicted flux ratio at PeV energies is consistent with that observed for bulge grammages ranging up to $\sim 100 \text{ g cm}^{-2}$. For grammages in the ADC lower than 2 g cm^{-2} , higher grammages in the bulges would be allowed.

We find that bulges in the outer rim of the accretion disk which are necessary to explain the asymmetric X-ray light curve observed in Cyg X-3 may provide target material for the production of γ rays at two distinct phases and that magnetic steering effects such as those inferred to take place in Hercules X-1 probably take place in Cyg X-3 as well.

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- White, N. E. & Holt, S. S. *Astrophys. J.* **257**, 318–337 (1982).
- Weekes, T. C. in *Proc. VIIth Astrophys. Meet. XX1st Rencontre de Moriond* (in the press).
- Watson, A. A. *Proc. 19th Int. Cosmic Ray Conf. (La Jolla)* **9**, (ed. Jones, F. C.) 111–140 (NASA, Washington DC, 1985).
- Protheroe, R. J. *Proc. astr. Soc. Astr.* **6**, 280–289 (1986).
- Eichler, D. *Nature* **275**, 725–726 (1978).
- Eichler, D. & Vestrand, W. T. *Proc. 16th Int. Cosmic Ray Conf. (Kyoto)* **1**, 147–149 (Institute for Cosmic Ray Research, Tokyo, 1979).
- Berezinsky, V. S. *Proc. DUMAND 1979 Summer Workshops at Khabarovsk and Lake Baikal* 245–261 (1979).
- Vestrand, W. T. & Eichler, D. *Astrophys. J.* **261**, 251–258 (1982).
- Hillas, A. M. *Nature* **312**, 50–51 (1984).
- Hillas, A. M. *Proc. 19th Int. Cosmic Ray Conf. (La Jolla)* **2** (ed. Jones, F. C.) 296–299 (NASA, Washington DC, 1985).
- Mason, K. O. & Cordova, F. A. *Astrophys. J.* **262**, 253–262 (1982).
- Shakura, N. I. & Sunyaev, R. A. *Astr. Astrophys.* **24**, 337–355 (1973).
- Hillas, A. M. *Proc. NATO Advanced Research Workshop on High Energy Gamma Ray Astronomy and Related Topics* (ed. Turver, K. E.) (Reidel, Dordrecht, in the press).
- van den Heuvel, E. P. J. & De Loore, C. *Astr. Astrophys.* **25**, 387–395 (1973).
- Petterson, J. A. *Accretion Discs in Close Binary Systems* (eds Lewin, W. G. H. & van den Heuvel, E. P. J.) 367–391 (Cambridge University Press, 1983).
- van der Klis, M. & Bonnet-Bidaud, J. M. *Astr. Astrophys.* **95**, L5–L7 (1981).
- Gorham, P. W. & Learned, J. *Nature* **323**, 422–424 (1986).
- Gorham, P. W. *et al. Astrophys. J.* **308**, L11–L15 (1986).
- Allen, C. W. *Astrophysical Quantities* (Athlone, London, 1973).
- Marcy, G. W. *IAU Symp. 102, Solar and Stellar Magnetic Fields: Origin and Coronal Effects* (ed. Stenflo, J. O.) 3–22 (Reidel, Dordrecht, 1983).
- Borra, E. F., Landstreet, J. D. & Mestel, L. A. *Rev. Astr. Astrophys.* **20**, 191–220 (1982).
- Channugam, G. & Brecher, K. *Nature* **313**, 767–768 (1985).
- Gaisser, T. K. *et al. Astrophys. J.* (in the press).
- Chadwick, P. M. *et al. Nature* **318**, 642–644 (1985).
- Baliunas, S. L. & Vaughan, A. H. A. *Rev. Astr. Astrophys.* **23**, 379–412 (1985).
- Lambert, A. *et al. Proc. 19th Int. Cosmic Ray Conf. (La Jolla)* **1** (ed. Jones, F. C.) 71–74 (NASA, Washington DC, 1985).
- Cawley, M. F. *et al. Astrophys. J.* **296**, 185–189 (1985).
- Lloyd-Evans, J. *et al. Nature* **305**, 784–787 (1983).
- Samorski, M. A. & Stamm, W. *Astrophys. J.* **268**, L17–L22 (1983).
- Protheroe, R. J. *Astr. Exp.* **1**, 33–38 (1984).

Cosmogenic ^{10}Be in Zaire alluvial diamonds: implications for ^3He contents of diamonds

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To determine the amounts of cosmic-ray produced ('cosmogenic') ^3He which could be created in diamonds during their post-eruptive residence in near-surface regions, we have measured the concentration of ^{10}Be (half life, 1.6 Myr) in an industrial diamond sample. The motivation to study cosmogenic ^3He in diamonds comes from the recent observations^{1,2} of $^3\text{He}/^4\text{He}$ ratios in some diamonds that are even higher than meteoritic values (so-called 'planetary' helium with $^3\text{He}/^4\text{He} = 1.4 \times 10^{-4}$). We measured ^{10}Be in an alluvial diamond sample, using radiochemical and accelerator mass spectrometry (AMS) techniques. The observed ^{10}Be concentration, $2.46 \pm 0.39 \times 10^6$ atoms g^{-1} , leads to an effective surface exposure of 3.3×10^5 yr for these diamonds. This corresponds to a substantial cosmogenic production of ^3He in the alluvial

diamonds, $\geq 29\%$ of the total ^3He . We conclude that the high $^3\text{He}/^4\text{He}$ ratios in at least some diamonds are due to cosmogenic ^3He production in near-surface regions during their residence in alluvial or weathering deposits. Thus, in order to establish that such high $^3\text{He}/^4\text{He}$ ratios existed in the mantle at one time, it is *a priori* necessary to study diamonds collected by underground mining methods.

In situ cosmogenic production of ^3He cannot be ruled out, because in the African diamonds analysed^{1,2}, samples were obtained from commercial sources, and it is known³ that alluvial diamonds represent about 80% of world production. Also, the Australian sample analysed by Honda *et al.*⁴ with a $^3\text{He}/^4\text{He}$ ratio of $157 \pm 75 \times 10^{-6}$ came from a 3-m-deep weathered deposit (C. B. Smith, personal communication). Production of ^3He due to cosmic ray and radiogenic thermal neutrons has recently been dealt with in detail⁵. Here we consider the *in situ* production of ^3He by cosmic ray spallation reactions in diamond.

One can calculate reasonably well the production rate of total spallation ^3He in the diamond (including that from the decay of ^3H) using the procedures followed earlier⁶ for determining isotope production rates at sea-level and underground. But the main uncertainty in determining the expected concentrations of ^3He resulting from *in situ* cosmic-ray reactions must be in the time and depth of burial. Cosmic-ray production of ^3He is important primarily in alluvial diamonds, or in diamonds extracted from weathered remains of the host rock. In diamonds extracted from rocks by underground mining at depths exceeding a few metres the cosmogenic production will arise primarily from fast mu-meson interactions⁵. This production rate drops by more than three orders of magnitude at pressures exceeding 3×10^3 g cm^{-2} (depth ~ 10 m). Any near-surface cosmic-ray production over periods of $\sim 10^6$ yr will then be more important than the maximum irradiation received underground during the entire age of the Earth.

Partly to circumvent uncertainties in depth and time of diamond irradiation, we decided to look for the spallation product ^{10}Be , which should be efficiently produced in spallation reactions on carbon. Principal production comes from ^{12}C spallation (threshold energy ~ 20 MeV). Spallation of ^{13}C , including the reaction $^{13}\text{C}(n, \alpha) ^{10}\text{Be}$ (threshold energy ~ 4 MeV), contributes only $\sim 1.5\%$ to total ^{10}Be production. The average yield of ^{10}Be in nuclear disintegrations in carbon has been estimated to be 2.5%⁷. Based on the published cross-sections for the formation of ^3H and ^3He from proton bombardment of carbon^{9,10}, and the star size distribution in 90 MeV neutron bombardment of carbon¹¹, we estimate, following earlier procedures⁸, that the average yields of ^3H and ^3He in C in cosmic-ray (neutron) stars will be 10 and 15% respectively. Similar values were obtained for disintegrations in air⁶. Thus, the production rate of ^{10}Be , relative to ^3He (total) is 0.1. The total rate of nuclear disintegration in carbon at any altitude and latitude can be obtained by multiplying the corresponding rates in the atmosphere by a factor of 0.88, which equals the ratio of the (geometric) nuclear cross-sections of carbon and air nuclei.

A sample of 10 g of industrial grade 16–18 grit diamond ('EMB') Diamond Abrasives Corporation, New York) was hand-picked for 'cloudy' specimens. The 'clear' and 'cloudy' diamonds were separately combusted in a stream of O_2 in a quartz tube, the output of which was connected to a bubbler containing 0.1 M HNO_3 with beryllium carrier having a $^{10}\text{Be}/^9\text{Be}$ ratio less than 3×10^{-15} . After combustion, the tube and bubbler were washed with carrier solution which was added to that in the bubbler. The chemical procedure for the extraction of ^{10}Be was the same as for meteoritic and lunar samples¹². The ^{10}Be measurements were performed on the University of Pennsylvania FN Tandem Van de Graaff accelerator¹³. The results are shown in Table 1.

A positive result was obtained for sample EMB-1, but only an upper limit could be obtained for EMB-2. However, if EMB-2

Table 1 ^{10}Be determinations in diamond samples

Sample code	Wt of sample (g)	Be carrier added (mg)	^{10}Be (10^6 atoms g^{-1})
EMB-1 (clear)	9.3	1.4	2.46 ± 0.39
EMB-2 (cloudy)	0.705	1.36	<6

had the same concentration as EMB-1 we would not expect a positive result because of the AMS background conditions.

Although our diamond sample was obtained from commercial sources, it was ascertained that the bulk of the sample was derived from Zaire alluvials. (In fact Zaire is the chief producer of industrial grade diamonds. In Zaire, the majority of the industrial diamonds derive from Mbuji-Mayi¹⁴.) The altitude of the alluvial deposits is 920 m, and the geomagnetic latitude is 5° S. The surface production rate at this site is calculated to be 16.0 ^{10}Be atoms g^{-1} diamond yr^{-1} . The observed ^{10}Be concentration of $2.46 \pm 0.39 \times 10^6$ atoms g^{-1} corresponds to $3.3 \pm 0.6 \times 10^5$ yr of effective surface exposure. (In this calculation, we assumed that only 50% of our sample was from the alluvium.) This can arise in several different ways, and it is important to consider plausible irradiation models in order to estimate the amount of ^3He produced *in situ* in the diamonds.

Clearly the diamonds have undergone a complex history of transportation and burial because of their extraction from the pipes upstream of the river bed. The exposure history of diamonds which have not been transported far from their point of origin (for example, diamonds in weathered kimberlite) should be simpler than that of diamonds in reworked alluvial deposits. We may for the sake of simplicity use two extreme models to characterize the two types of deposits.

In model 1 the diamonds remained at some mean depth x_b , the effective depth of burial, throughout their history. In model 2 it is assumed that the diamonds spend equal times at different depths from the land surface to a depth x_m , the depth of mixing. Model 1 assumes no transportation, whereas model 2 is applicable to the case of extensive alluvial deposits. The mean depths x_b and x_m are related to the observed concentration of ^{10}Be in the diamond, C (atoms g^{-1}), and the surface production rate, P_0 (atoms $\text{g}^{-1} \text{s}^{-1}$), by the following equations:

$$C(1) = \frac{P_0}{\lambda} e^{-\rho x_b / \Lambda} \quad (1)$$

$$C(2) = \frac{P\Lambda}{\lambda \rho x_m} (1 - e^{-\rho x_m / \Lambda}) \quad (2)$$

In equations (1) and (2) the *in situ* cosmic-ray production rate of ^{10}Be is assumed to vary with a mean path length of Λ g cm^{-2} ; ρ is the mean density of the host material. The calculated values of x_b and x_m are 1.12 ± 0.10 and 4.25 ± 0.70 m respectively for the observed ^{10}Be value (Table 1) with $\Lambda = 150$ g cm^{-2} . Equations (1) and (2) assume continuous irradiation until the time of collection of the diamonds; if this is not the case the calculated depths represent overestimates.

For several reasons the expected amount of *in situ* cosmogenic ^3He may significantly exceed the calculated factor of ten times the ^{10}Be production. The ^{10}Be concentration reaches a steady-state equilibrium value for a fixed geometry, whereas ^3He will continue to accumulate in the diamond. If the last irradiation were at a deeper depth, we would underestimate the ^3He production rate because of the decay of some ^{10}Be . Thus it is actually possible to have cosmogenic ^3He but no ^{10}Be .

The minimum amount of cosmogenic ^3He expected in the Zaire alluvial diamonds is then obtained by multiplying the 'effective' ^{10}Be surface exposure time by the total ^3He production rate at the site: 1.6×10^2 atoms g^{-1} diamond yr^{-1} . This value is $5.3 \pm 1.0 \times 10^7$ atoms g^{-1} of ^3He or ~ 2 $\mu\text{cm}^3 \text{g}^{-1}$ of ^3He .

Table 2 gives the ^3He and ^4He concentrations and ratios in Zaire diamonds measured by us, and also those reported in the

Table 2 ^3He and ^4He concentrations in Zaire diamond samples

Sample code	Concentration*		R/R_A	Reference
	^3He ($\mu\text{cm}^3 \text{g}^{-1}$)	^4He ($\mu\text{cm}^3 \text{g}^{-1}$)		
EMB	11.5	3.5	2.4	Present work†
Zaire-1	7.01	2.0	2.5	Present work‡
830204	6.64	1.39	3.4	Ozima <i>et al.</i> ²
830805	7.65	1.74	3.1	Ozima <i>et al.</i> ²
ZAI	6.8	0.98	4.9	Honda <i>et al.</i> ⁴

$^3\text{He}/^4\text{He}$ ratios expressed as R/R_A where R_A , the atmospheric ratio is 1.4×10^{-6} .

* Errors on individual $^3\text{He}/^4\text{He}$ measurements are <10%, except in the case of ZAI where the errors on the ^3He and ^4He concentrations and the ratio are ± 33.8 , 14.3 and 33.3% respectively.

† EMB industrial grade diamonds, the bulk of which are from Zaire (G. van Maarseveen), personal communication).

‡ Sample obtained from Dr Henry Gaillard, Michael Werdiger, Inc. New York.

literature^{2,4}. We note that the EMB ^3He and ^4He concentrations measured by us are 70% greater than the 'pure' Zaire concentrations, but the $^3\text{He}/^4\text{He}$ ratios are quite consistent with the other values for Zaire diamonds. Since the EMB sample is a conglomerate, we assume the Zaire-1 isotope concentrations in what follows. It then follows from the ^{10}Be data that at least $\sim 2/7$, that is >29% of the ^3He in Zaire diamonds is cosmogenic in origin. Ozima *et al.*¹ analysed helium in 38 diamonds from various sources. Nine of these samples have ^4He concentrations exceeding 1 $\mu\text{cm}^3 \text{g}^{-1}$ of ^4He and $^3\text{He}/^4\text{He}$ ratios with $R < R_A$ (where $R = ^3\text{He}/^4\text{He}$ and R_A is the atmospheric ratio), except for two Zaire diamonds which have ratios of 3.1 and 3.4 R_A . This observation is consistent with our conclusion that most of the He in Zaire diamonds is probably cosmogenic in origin.

What may one expect for the upper limit of cosmogenic contributions in individual diamonds from alluvial sources? This is a difficult question. Let us rather examine whether the observed concentrations in diamonds with anomalously high $^3\text{He}/^4\text{He}$ ratios are consistent with cosmogenic production.

Ozima *et al.*^{1,2} found $^3\text{He}/^4\text{He}$ ratios in four samples with $R > 50 R_A$: the observed ^3He concentrations are 8.7, 42, 44 and 54 $\mu\text{cm}^3 \text{g}^{-1}$ and the corresponding ratios 167, 225, 58 and 84 R_A . Honda *et al.*⁴ observed two such samples with 7.6 and 24 $\mu\text{cm}^3 \text{g}^{-1}$ of ^3He and corresponding ratios of 112 ± 53 and $58 \pm 13 R_A$. The lower bound of these ^3He concentrations is about the same as observed in Zaire diamonds, that is, 7 $\mu\text{cm}^3 \text{g}^{-1}$. The upper bound is only 7.7 times larger. We believe that most of this excess ^3He has been produced cosmogenically. The irradiation times required to produce the $\sim 45 \mu\text{cm}^3 \text{g}^{-1}$ excess ^3He in the three samples analysed by Ozima *et al.* are 7.5 Myr for surface exposure, and 16 and 68 Myr respectively for variable depth exposure (model 2), with mean mixing depths of 1 and 5 metres. The EMB sample cosmic-ray irradiation corresponds to a secular equilibrium exposure for ^{10}Be (5 Myr) for a mean mixing depth of 4.25 m (model 2). This is the sample-averaged irradiation time; in fact we expect large, probably at least order of magnitude, differences in the irradiation times of individual diamonds, considering their possibly complex and variable exposure history. The eruption ages of diamond-bearing kimberlites show a wide spread from 50 Myr to 1,500 Myr; the latter is the time during which the diamondiferous areas of stable cratons have not undergone an orogenic phase¹⁵. Cosmogenic ^3He cannot therefore be ruled out as the origin of the anomalously high $^3\text{He}/^4\text{He}$ ratios in the three undocumented samples of Ozima *et al.*^{1,2}.

Finally, we comment on the high $^3\text{He}/^4\text{He}$ ratios observed in two diamonds from the Australian Argyle lamproite deposits⁴. As mentioned earlier, these diamonds were not collected by underground mining methods, but were recovered from a pit

containing about 3-m depth of weathered host rock (C. B. Smith, personal communication). We note that the excess ^3He in these diamonds is only $\sim 8 \mu\text{m}^3 \text{g}^{-1}$ of ^3He . Therefore, we still do not know of a single case of high $^3\text{He}/^4\text{He}$ ratios in a diamond which has been well-shielded from cosmic radiation throughout its history.

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1. Ozima, M. *et al. Geochim. cosmochim. Acta* **47**, 2217 (1983).
2. Ozima, M. *et al. Geochim. J.* **19**, 127–134 (1985).
3. Bruton, E. *Diamonds* (Chilton, Philadelphia, 1971).
4. Honda, M., Reynolds, J. H., Roedder, E. & Epstein, S. *J. geophys. Res.* (in the press).
5. Lal, D. *Isotope Geoscience* (in the press).
6. Lal, D. & Peters, B. *Handbuch der Physik* XLVI/2, 551–612 (Springer, Berlin, 1967).
7. Lal, D. *et al. Meteoritics* **20**, 403–414 (1985).
8. Lal, D. thesis, Tata Inst. Fundamental Research, Bombay (1958).
9. Honda, M. & Lal, D. *Phys. Rev.* **118**, 1618–1625 (1960).
10. Bailey, L. E. thesis, Univ. California, Berkeley (1956).
11. Kellogg, D. A. *Phys. Rev.* **93**, 1337–1339 (1953).
12. Nishiizumi, K. *et al. Earth planet. Sci. Lett.* **70**, 157–163 (1984).
13. Klein, J. *et al. Nucl. Instrum. Meth.* **193**, 610–616 (1982).
14. Legrand, J. *Diamonds, Myth, Magic and Reality* (Crown, New York, 1980).
15. Dawson, J. B. *Kimberlites and their Xenoliths* (Springer, Berlin, 1980).

Noble-gas enrichment in vapour-growth diamonds and the origin of diamonds in ureilites

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Ureilites show high carbon contents comparable with those of CM chondrites^{1,2}. One of the major questions about ureilites is why they contain large amounts of noble gases concentrated in carbon-rich veins^{3,4}. Diamond is shown to be one of the noble-gas carriers, while graphite is gas-free⁵. We synthesized diamonds by chemical vapour deposition (CVD)^{6–8} from gaseous mixtures of H_2 and CH_4 including Ar, and examined the Ar trapped in diamond by mass spectrometry employing the stepwise heating technique. The partial pressure of ^{36}Ar in the gaseous mixture during the synthesis of diamond was 5.3×10^{-6} atm. The content of ^{36}Ar was about as great as $8 \times 10^{-6} \text{ cm}^3 \text{STP g}^{-1}$ which was extracted at 2,000 °C. Meanwhile, the ^{36}Ar concentration in graphite was much less than 5% of that in diamonds. These results suggest that diamonds in ureilites may have been directly formed from the solar nebula.

A schematic drawing of the experimental apparatus is shown in Fig. 1. Gases were evacuated by a rotary pump and fed into a quartz discharge tube in a microwave oven. A mixture of CH_4 and H_2 gases (concentrations of CH_4 of 0.5, 1 and 5% (v/v)) was used, and Ar corresponding to 4% of these gaseous mixtures was used in some cases to study its incorporation during diamond deposition. Gas concentrations were determined only by the flow rate. The total pressures were 30 torr except for one experiment performed at 20 torr. The discharge was maintained for a period of ~ 20 h. The carbon products were deposited on scratched silicon wafer substrates in the quartz tube. By dissolving the silicon wafers in acid solution ($\text{HF}:\text{HNO}_3 = 5:1$), the carbon products could be isolated. The synthesis parameters and the results of X-ray diffraction analyses are shown in Table 1. Nothing except diamond or graphite was detected in the X-ray

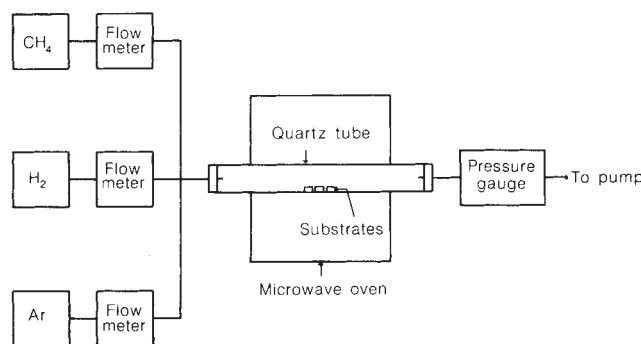


Fig. 1 Schematic diagram of the experimental apparatus of diamond synthesis.

diffraction lines. But no information on the abundance of amorphous carbon could be obtained by this method. As obvious from Table 1, high CH_4 concentrations seem to favour the growth of graphite, a fact also reported by other researchers⁹. According to the optical microscopic observations, carbon products produced in the Ar-containing atmosphere seemed to be the same as that in Ar-free conditions as long as the CH_4 concentration was the same. But although samples 14 and 8 were formed using identical CH_4 concentrations, the lines of diamond detected in sample 14 were faint only. This suggests that the addition of Ar impeded the deposition of diamond.

Six samples, numbers 4, 8, 11, 13, 15 and 14 were analysed for trapped ^{36}Ar by mass spectrometry using the stepwise heating technique. Samples 13 and 15 were essentially identical, and were prepared to check the reproducibility. At each step the temperature of the sample was held constant for 30 min to extract gases, and then was reduced to room temperature. For the samples without Ar (4 and 8), no 2,100 °C step was performed because of their low yield at 2,000 °C. Although only ^{36}Ar was measured for most samples, the $^{40}\text{Ar}/^{36}\text{Ar}$ ratios were measured for samples 14 and 15 in the 800 and 2,000 °C fractions. They were indistinguishable from the atmospheric value of about 3×10^2 . The uncertainty in the amounts of ^{36}Ar was less than 10%. Because of the small mass of samples, errors in weighing (approximately <20 to 30%) are the major source of errors in the concentration of ^{36}Ar . Blank corrections were applied to all data. The results are given in Table 1. Where the blank corrections exceeded 10%, the percentage of the blank relative to the measured value is shown in parentheses. For the samples synthesized in the Ar atmosphere they were negligible for most temperature fractions. In Fig. 2 the release patterns of ^{36}Ar for the samples 11, 13 and 15 show the major release (85%) to be at the 2,000 °C fractions. Compared with these, diamond formed with no Ar admixed (sample 4) contains much less ^{36}Ar (<1%). It can be ruled out therefore, that the ^{36}Ar in samples 11, 13 and 15 was adsorbed from the terrestrial atmosphere at some stage. Their Ar must have been tightly trapped when they were synthesized by vapour growth. Samples 13 and 15 seem to have a small secondary release peak at 1,400 °C. It is not clear whether these small peaks originate from another carbon phase such as amorphous carbon rather than diamond. Sample 14 is composed mainly of graphite with a slight amount of diamond. The concentration of ^{36}Ar at the 2,000 °C fraction for sample 14 provides an upper limit of ^{36}Ar concentration from graphite, which amounts to only 5% of the concentrations in samples 11, 13 and 15. The release pattern of sample 14 looks flat for the lower temperature fractions, which is also different from that for samples 11, 13 and 15. Our results are consistent with the observation by Göbel *et al.*⁵ that graphite can be excluded as a carrier of noble gases in ureilites. We have interpreted these similarities to indicate that noble gases in ureilite diamonds

Table 1 Parameters for the CVD process and the results of X-ray diffraction and mass spectrometry

Sample number	Parameters				Time (h)	X-ray diffraction†		Sample weight (mg)	Mass spectrometry	
	Gas composition*			Total pressure (torr)		Diamond	Graphite		Temp. (°C)	$^{36}\text{Ar}^\ddagger$ ($\times 10^{-8} \text{ cm}^3 \text{ STP g}^{-1}$)
7	CH ₄ (%)	H ₂ (%)	Ar (%)							
6	0.5	99.5	0	30	20	+	—		ND	
	1	99	0	20	21	+	—		ND	
									800	0.32 (48%)
									1,100	1.8 (14%)
4	1	99	0	30	26	+	—	2.0	1,400	1.1 (21%)
									1,700	1.2 (19%)
									2,000	1.3 (36%)
									800	1.7
									1,100	0.57 (11%)
8	5	95	0	30	21	+	+	7.9	1,400	0.44 (14%)
									1,700	0.55 (12%)
									2,000	0.083 (70%)
									800	0.5 (47%)
									1,100	10
11	0.5	95.5	4	30	20	+	—	1.3	1,400	19
									1,700	35
									2,000	750
									2,100	4.0 (23%)
									800	0.53 (24%)
									1,100	13
									1,400	62
13	1	95	4	30	21	+	—	3.4	1,700	19
									2,000	880
									2,100	30
									800	0.32 (42%)
									1,100	2.3 (12%)
15	1	95	4	30	24	+	—	5.2	1,400	16
									1,700	10
									2,000	810
									2,100	12
									800	3.4
									1,100	5.3
14	5	91	4	30	20	+	+	15.5	1,400	4.9
									1,700	3.6
									2,000	40
									2,100	2.3

* Error of gas content is 20%.

† Present (+); absent (—). The content of diamond in sample 14 should be much smaller than that of graphite judging from the intensity of the diffraction lines.

‡ The blank correction to the measured value is given in parentheses in case it exceeded 10%. ND = not determined.

were trapped by similar mechanisms and that these diamonds were deposited directly from the solar nebula.

The major element of the solar nebula was hydrogen¹⁰. Although CO is the dominant carbon-bearing compound at high temperature, it progressively transforms to CH₄ on cooling according to the reaction $\text{CO} + 3\text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O}$ ¹⁰. Therefore, the condition of the gaseous mixture to form diamonds could have been satisfied in the solar nebula. The reverse zoning at the grain boundary of ureilite olivines is generally thought to be formed as a result of reduction by the matrix^{11,12}. If the carbon material in ureilites had directly formed from the hydrogen atmosphere, it may be possible that the reduction of olivines also occurred at that time.

The noble gases trapped in ureilites show the low $^{40}\text{Ar}/^{36}\text{Ar}$ ratio ($<10^{-3}$) and the correlation of their elemental abundance ratios with the respective ionization energies⁵. These features impose constraints on models of ureilite genesis. The low $^{40}\text{Ar}/^{36}\text{Ar}$ ratio is incompatible with the hypothesis that the noble gases and their carriers took part in geological processes on a parent body for an extended period of time¹³. The correlation of elemental abundance of noble gases with the ionization energies could be explained quite naturally if noble gases were trapped by a plasma process. Recently, Lewis *et al.*¹⁴ reported the existence of diamonds even in Allende, Murchison and

Indarch, which also suggested the primary formation of diamond by stellar condensation.

Large amounts of noble gases were also contained in diamond-free ureilite, which means the occurrence of another noble-gas carrier like amorphous carbon^{15,16}. If the secondary release peak of our samples 13 and 15 at 1,400 °C is due to another carbon phase rather than diamonds, this phase could be another noble gas carrier. Otherwise, it is probable that very fine grains ($<50 \text{ \AA}$) of diamonds could not be detected by X-ray diffraction. The recent discovery that most grains of Allende carbon residue were diamonds was identified by the electron diffraction method although the X-ray diffraction lines were very faint.

Assuming the total solar nebula pressure as 10^{-3} atm ¹⁰, a partial pressure of ^{36}Ar in the solar nebula calculated from Solar System abundances¹⁷ is $5.5 \times 10^{-9} \text{ atm}$. The concentration of ^{36}Ar trapped in diamond by vapour growth in the solar nebula can be estimated if it is proportional to the partial pressure of ^{36}Ar . Here the partial pressure of ^{36}Ar was $5.3 \times 10^{-6} \text{ atm}$. By assuming that the concentration of trapped ^{36}Ar is $8.8 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$, the highest concentration (sample 13 at the 2,000 °C fraction) obtained here, the concentration of ^{36}Ar trapped in diamond synthesized in the solar nebula is estimated to be $9.1 \times 10^{-9} \text{ cm}^3 \text{ STP g}^{-1}$. This value is still three to four orders of magnitude smaller than the concentration of ^{36}Ar trapped in

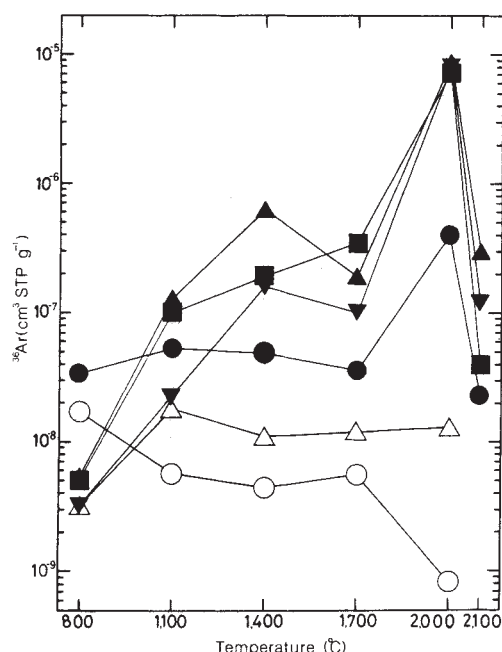


Fig. 2 Release patterns of ^{36}Ar from the different samples synthesized in Ar atmosphere (black symbols) and the samples in an Ar-free atmosphere (open symbols) upon stepwise heating. Methane content is 0.5% (squares), 1% (triangles) and 5% (circles), respectively. Sample numbers, 4, Δ ; 8, $\square$; 11, $\blacksquare$; 13, $\blacktriangle$; 15, $\blacktriangledown$ and 14, $\bullet$.

the diamonds in ureilites⁵, however it is four to five orders of magnitude larger than the concentration of ^{36}Ar that can be expected to be trapped by other mechanisms such as the condensation of some metal and oxides¹⁸ or solubility in basalt melt¹⁹. That the estimated concentration of ^{36}Ar here is lower than that of ureilites, may have resulted from the fact that the concentration of Ar trapped in diamonds may not be proportional to the partial pressure of Ar. It is conceivable that Ar concentrations in synthesized diamonds in this study are already saturated because the partial pressure of Ar is high. It is expected that the growth rate of diamond was different between sample 11 (CH_4 concentration, 0.5%) and samples 13, 15 (CH_4 concentration, 1%). The ^{36}Ar concentrations at 2,000 °C are almost identical for these three samples, suggesting that the growth rate of diamond has no relation to the trapping mechanism of noble gases.

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1. Vdovikin, G. P. *Space Sci. Rev.* **10**, 483–510 (1970).
2. Grady, M. M., Wright, I. P., Swart, P. K. & Pillinger, C. T. *Geochim. cosmochim. Acta* **49**, 903–915 (1985).
3. Weber, H. W., Hintenberger, H. & Begemann, F. *Earth planet. Sci. Lett.* **13**, 205–209 (1971).
4. Weber, H. W., Begemann, F. & Hintenberger, H. *Earth planet. Sci. Lett.* **29**, 81–90 (1976).
5. Göbel, R., Ott, U. & Begemann, F. *J. geophys. Res.* **83**, 855–867 (1978).
6. Derjaguin, B. V. *et al. J. Crystal Growth* **2**, 380–384 (1968).
7. Matsumoto, S., Sato, Y., Kamo, M. & Setaka, N. *J. appl. Phys.* **21**, L183–L185 (1982).
8. Shindo, H., Miyamoto, M., Matsuda, J. & Ito, K. *Meteoritics* **20**, 754 (1985).
9. Sato, Y., Matsumoto, S., Kamo, M. & Setaka, N. *J. Surface Sci. Soc. Jap.* **5**, 54–60 (1984).
10. Grossman, L. & Larimer, J. W. *Rev. Geophys. Space Phys.* **12**, 71–101 (1974).
11. Berkley, J. L. *et al. Geochim. cosmochim. Acta* **40**, 1429–1437 (1976).
12. Miyamoto, M., Takeda, H. & Toyoda, H. *J. geophys. Res.* **90**, 116–122 (1985).
13. Begemann, F. & Ott, U. *Geochim. cosmochim. Acta* **47**, 975–977 (1983).
14. Lewis, R. S., Ming, T., Wacker, J. F. & Steel, E. *Lunar planet. Sci. XVIII* (in the press).
15. Ott, U., Löhr, H. P. & Begemann, F. *Meteoritics* **19**, 287–288 (1984).
16. Wacker, J. F. *Geochim. cosmochim. Acta* **50**, 633–642 (1986).
17. Anders, E. & Ebihara, M. *Geochim. cosmochim. Acta* **46**, 2363–2380 (1982).
18. Honda, M., Ozima, M., Nakada, Y. & Onaka, T. *Earth planet. Sci. Lett.* **43**, 197–200 (1979).
19. Jambon, A., Weber, H. & Braun, O. *Geochim. cosmochim. Acta* **50**, 401–408 (1986).

Planar OH-bearing defects in mantle olivine

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Olivine, $(\text{Mg}, \text{Fe})_2\text{SiO}_4$, is the predominant mineral in the upper mantle and a study of its defect structure is fundamental to an understanding of the rheological laws that describe mantle flow. Existing models are based on creep mechanisms in which point and line defects have a major role¹. Here we report the first observations, by transmission electron microscopy (TEM), of planar defects in olivine. The displacement vector associated with the defects, $\mathbf{R} = 1/4\langle 011 \rangle$, together with infrared absorption spectra suggest that the structure of the defects resembles that of an OH-bearing monolayer within the olivine, as exists in the humite family of minerals. Because the planar defects were formed in the upper mantle, their discovery has important implications for the deformation of olivine at high pressures and temperatures in the presence of trace quantities of water, as well as revealing another possible reservoir of water in the upper mantle.

Several single crystals of olivine, millimetres in size, were collected from a kimberlite in Buell Park, Arizona, USA. The petrographic features of this kimberlite are rather similar to the type kimberlites found in South Africa and Lesotho, although phlogopite and diamond are absent in the former while titanite clinohumites, titanite chondrodites and amphiboles are present². Most of the olivine crystals are pale green but some are orange in colour. All of the crystals in thin section were homogeneous in composition (Fo_{90}) and show no specific textures under an optical microscope other than rods of hydrated zones observed in some crystals.

TEM shows that all of the green crystals have only dislocation microstructures³. The orange crystals, however, have large regions with no dislocations but with planar defects both on $\{001\}$ and $\{021\}$ planes and occasional serpentine zones on $\{010\}$, cutting across the defects (Fig. 1a and b). The planar defects on $\{001\}$ are common and some of them terminate at partial dislocations. Those on $\{021\}$ are generally shorter than on $\{001\}$ and appear in zigzag lines consisting of the $\{021\}$ and $\{001\}$ faults (Fig. 1b). Most of the planar defects appear as isolated monolayers. But some defects in $\{001\}$ appear as a band consisting of a few adjacent defects; bands of two defects are common, three rather rare, and thicker bands very rare. The density of the defects is roughly a few per micrometre. No special concentration of elements has been observed by analytical TEM. Using $\mathbf{g} \cdot \mathbf{R}$ criteria in diffraction contrast imaging (that is, the defects are invisible when imaged with a diffracted beam with reciprocal lattice vector $\mathbf{g}$ if the dot product $\mathbf{g} \cdot \mathbf{R}$ is an integer) the displacement vector $\mathbf{R}$ of the defects is $\mathbf{R} = 1/4\langle 011 \rangle$. Direct lattice imaging (Fig. 2) confirms this value of the displacement vector.

As the displacement vector is not parallel to the plane of the defects they should change the olivine stoichiometry. The structure of the defects may be determined directly by a comparison of the crystal structures of olivine and the humite family of minerals⁴. The structures of the humite group can be looked upon as being composed of alternate olivine and brucite-like slabs whose compositions may be expressed as $\text{Mg}_{2n-1}\text{Si}_n\text{O}_{4n-2}$ and $2\text{Mg}(\text{OH})\text{O}$ (ref. 5). In the clinohumite structure $n = 4$. The olivine slabs separated by the $2\text{Mg}(\text{OH})\text{O}$ slab are displaced relative to one another by the vector $\mathbf{R} = 1/4\langle 011 \rangle$, in other words

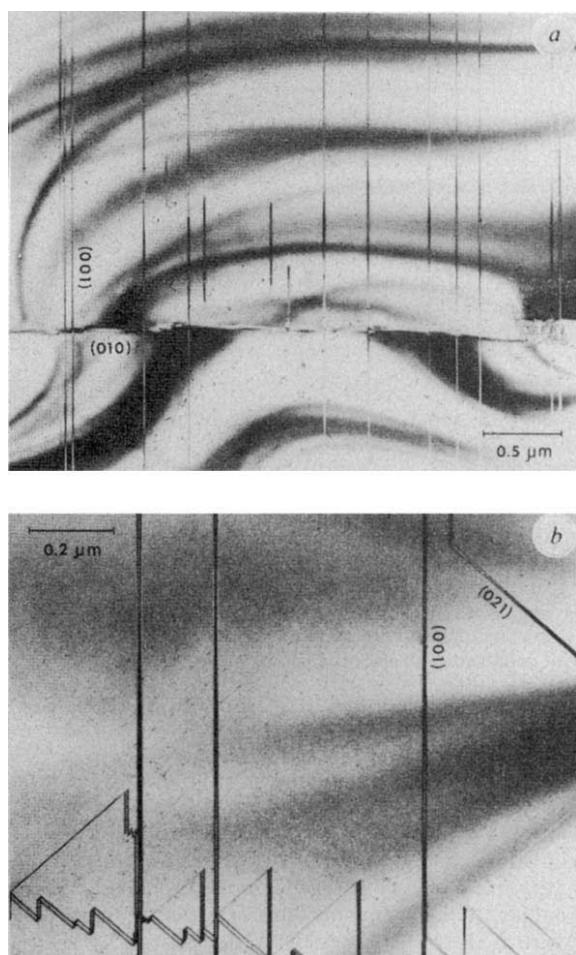


Fig. 1 Low-magnification electron micrographs of planar defects in olivine from a kimberlite in Buell Park. *a*, The planar defects on {001} are common and some of them terminate at partial dislocations. Occasional serpentine zones on {010} cut across the defects. *b*, The defects on {021} appear in zigzag lines.

the hydroxyl-bearing slab acts like a planar defect with $\mathbf{R} = 1/4\langle 011 \rangle$ (Fig. 3). High-resolution lattice imaging supports the interpretation that the planar defects observed on both {001} and {021} planes in the olivine described here are of this type. Where defects are adjacent their shortest spacing is commonly the same as that in clinohumite, further supporting this interpretation.

The OH-bearing composition of the defects is confirmed by infrared absorption spectroscopy. The infrared absorption spectra of one of the crystals with the planar defects has been studied in [100], [010] and [001] polarizations (Fig. 4). These spectra clearly demonstrate that this olivine has several hydroxide components. The total OH concentration is $5.1 \mu\text{mol cm}^{-3}$, calculated using the molar absorptivity correction⁶. The polarization dependence indicates that the OH dipoles are strongly orientated. No broadband component is evident near the $3,420 \text{ cm}^{-1}$ characteristic wavenumber of the molecular water absorption.

The $3,675 \text{ cm}^{-1}$ band in the olivine spectra is due to serpentine. The $3,610$, $3,598$, $3,477$ and $3,230 \text{ cm}^{-1}$ bands, found in infrared spectra of olivines from many localities, are believed to be due to OH defects in the olivine lattice⁷. Superimposing the spectra upon a spectrum of titanian clinohumite from Moses Rock shows the correspondence in position and width of the infrared bands at $3,571$, $3,524$, $3,402$ and possibly at $3,319 \text{ cm}^{-1}$. In addition the $3,402 \text{ cm}^{-1}$ band is only a minor component in the spectrum of magnesian clinohumite. Its prominence in the Buell

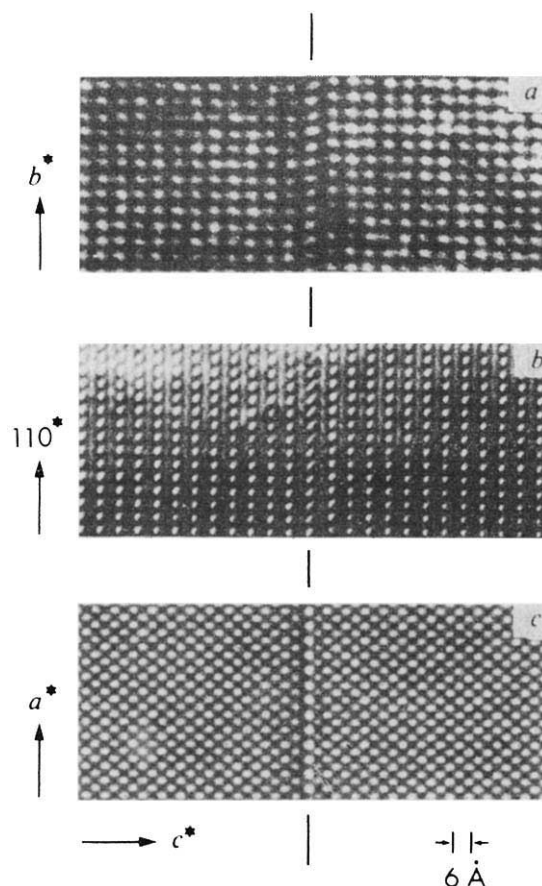


Fig. 2 Lattice images of the planar defects in {001} on *a*, ($b^* - c^*$); *b*, ($110^* - c^*$); *c*, ($a^* - c^*$) planes. The displacement vector of the planar defects $\mathbf{R} = 1/4\langle 011 \rangle$.

Park olivine spectrum suggests that this clinohumite is titanian. The pleochroism of this sample, ($E \parallel b$) = strong orange, and ($E \parallel c$) = ($E \parallel a$) is strong orange yellow, is also similar to that of titanian clinohumite⁷. The spectroscopic analysis therefore supports the structural interpretation from the TEM study and indicates that the defects are essentially monolayers of titanian clinohumite within the olivine.

The hydration of olivine forms minerals such as serpentine and humite family minerals, depending on the pressure and temperature conditions. Clinohumite and chondrodite among the humite family minerals are stable under the pressure and temperature conditions of the upper mantle in the presence of water⁸. The genetic relations between the serpentine zones and the defects indicate that the defects formed before the formation and above the stability field of serpentine. The coexistence of mantle derived titanian clinohumite and clinochondrodite in this kimberlite², and the kimberlite in the same plateau⁹ also suggests that the planar defects with titanium component have been formed in the upper mantle.

Most of the olivine crystals in the kimberlite have dislocation microstructures and no planar defects, whereas those parts of crystals containing planar defects are devoid of any dislocations apart from the partials at the terminations of the defects. While the present observations do not confirm the origin of the defects, three possible interpretations can be given: (1) by precipitation of OH-bearing monolayers from 'hydrous olivine', (2) by hydration of olivine within the stability field of humite family minerals, and (3) formed in a dislocation-free crystal if during the deformation process glide of partial dislocations generating planar defects is energetically preferred as a mechanism of deformation in the presence of sufficient water.

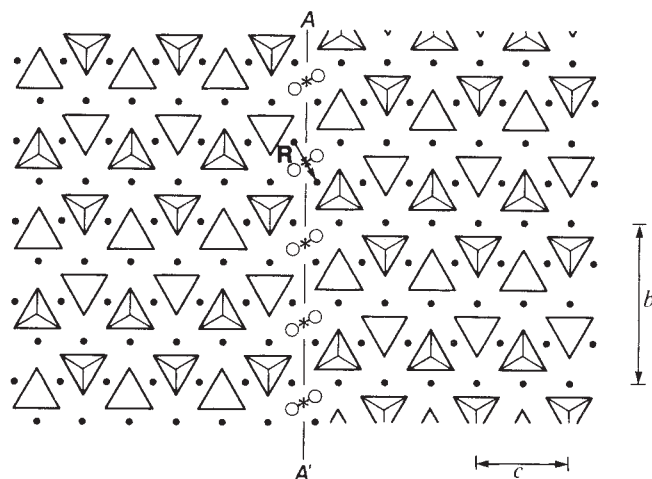


Fig. 3 Schematic drawing of the structure of the planar defect (A-A') based on the lattice images in Fig. 2a and the known structure of humite family minerals. The triangles show SiO_4 tetrahedra and solid circles show (Mg,Fe) atoms. Open circles and asterisks represent oxygen atoms not bonded to tetrahedra and hydrogen atoms, respectively. The displacement vector across the planar defect $\mathbf{R} = 1/4[011]$.

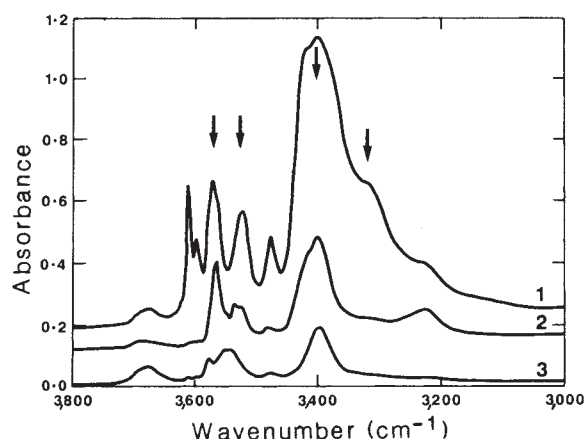


Fig. 4 OH absorption spectra for the olivine. The spectra 1, 2 and 3 are polarized [100], [001] and [010] respectively. The sample thickness for the spectra were normalized to 1.0 mm to facilitate direct comparison. The polarization dependence indicates strongly orientated OH dipoles. The bands at 3,571, 3,524 and 3,402 and possibly at 3,319 cm^{-1} , indicated by arrows, can be assigned to titanian clinohumite. The 3,675 cm^{-1} band is due to serpentine. The 3,610, 3,598 and 3,230 cm^{-1} bands can be assigned to OH point defects in the olivine lattice.

If the existence of planar defects in mantle olivine proves to be a general phenomenon (titanian clinohumite infrared bands have also been found in olivines from the Monastery kimberlite, South Africa and from Zabargad Island, Egypt⁷), current theories on the deformation of the upper mantle, where the role of water has been recently stressed¹⁰, will need to be substantially revised. Creep experiments under wet conditions have been carried out at lower pressure and temperature conditions than in the upper mantle as it has been assumed that extrapolation to mantle conditions would not introduce any change in the deformation mechanism. The present findings cast doubts on this assumption. A further implication of the present work is that water in the mantle may be incorporated in olivine as well as in other nominally anhydrous minerals (for example, garnet¹¹) in addition to hydrous minerals such as phlogopite or titanian clinohumite.

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1. Poirier, J. P. *Creep of Crystals* (Cambridge University Press, 1985).
2. Aoki, K., Fujino, K. & Akaogi, M. *Contrib. Miner. Petrol.* **56**, 243-253 (1976).
3. Kitamura, M., Matsuda, H. & Morimoto, N. *Proc. Jap. Acad.* **62**, 149-152 (1986).
4. Bragg, W. L. & Claringbull, G. F. *The Crystalline state, vol IV: Crystal structures of minerals* (Bell, London, 1965).
5. Fujino, K. & Takeuchi, Y. *Am. Miner.* **63**, 535-543 (1978).
6. Paterson, M. S. *Bull. Miner.* **105**, 20-29 (1982).
7. Miller, G. H., Rossman, G. R. & Harlow, G. E. *Phys. Chem. Minerals* (in the press).
8. Yamamoto, K. & Akimoto, S. *Am. J. Sci.* **277**, 288-312 (1977).
9. McGetchin, T. R., Silver, L. T. & Chodos, A. A. *J. geophys. Res.* **75**, 255-259 (1970).
10. Karato, S., Paterson, M. S. & FitzGerald, J. D. *J. geophys. Res.* **91**, 8151-8176 (1986).
11. Aines, R. D. & Rossman, G. R. *Geology* **12**, 720-723 (1984).

Microwave absorption in aqueous solutions of DNA

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In a recent *Nature* leading article¹ the possibility of the occurrence of resonance absorption at microwave frequencies in DNA was discussed in terms of the latest available theoretical² and experimental evidence³, and its implications were assessed. Because of the far-reaching nature of these implications it is important that the existence or otherwise of such absorption be firmly established. Here we report on a concerted effort in two independent laboratories which has involved the measurement of the dielectric properties of aqueous solutions of circular DNA molecules in the frequency range 1-10 GHz. No resonance absorption or any form of enhanced absorption was demonstrated.

The theoretical work underlying this phenomenon dates from Prohofsky, Van Zandt and their associates who developed a model of electromagnetic energy coupling through acoustic vibrations along the axis of helical polymers⁴. The mechanism is applicable to DNA molecules and predicts high-absorption cross-sections at microwave frequencies. The frequency dependence and damping parameter of these absorptions are determined by the nature and degree of interaction between solute and solvent molecules^{5,6}. More recently, Scott⁷ applied a non-linear soliton approach to this problem by assuming the excited acoustic wave to be anharmonic. Both theories evolved by drawing heavily on the experimental evidence provided by Edwards, Swicord and Davis³ of resonance absorption by circular and linear segments of DNA molecules in aqueous solutions.

To ensure a proper comparison with the data published by Edwards *et al.*^{3,8} we used the same form of DNA, that is, the DNA plasmid pUC8.c2 of 5,480 base pairs (bp), which is a dimer of pUC8 (2,740 bp). The material was isolated from *Escherichia coli* HB101 by standard plasmid procedures and dissolved in 10 mM Tris, 10 mM NaCl, 1 mM EDTA at pH 7.5 to make 0.1% w/w plasmid solutions. Only preparations with an optical density of 260/280 ratio between 1.9 and 2 were used.

The dielectric measurements in London were performed on an automated time domain spectrometer using a reflection technique. The measurements in Uppsala were carried out on a similar spectrometer but with a transmission technique. Full experimental details have appeared previously^{9,10}. A common and most important feature in the experimental procedure is the use of a reference sample to normalize the measured reflection or transmission coefficient thus eliminating any systematic experimental artefacts which may arise, for example, from a

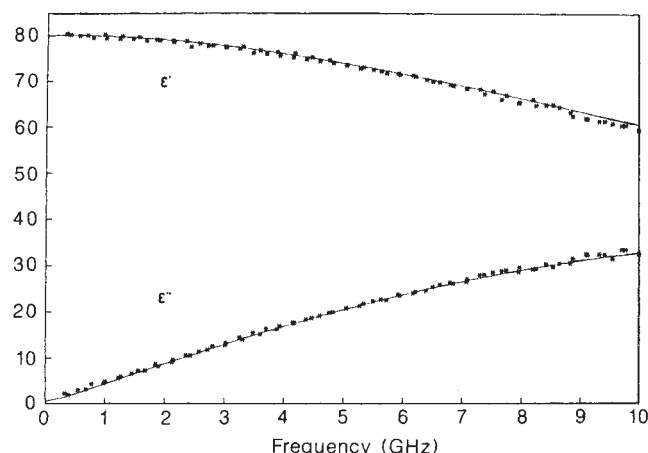


Fig. 1 Relative permittivity (ϵ') and loss factor (ϵ'') of 0.1% plasmid DNA solution at 20 °C. (The full lines are the literature values of the dielectric parameters of pure water.)

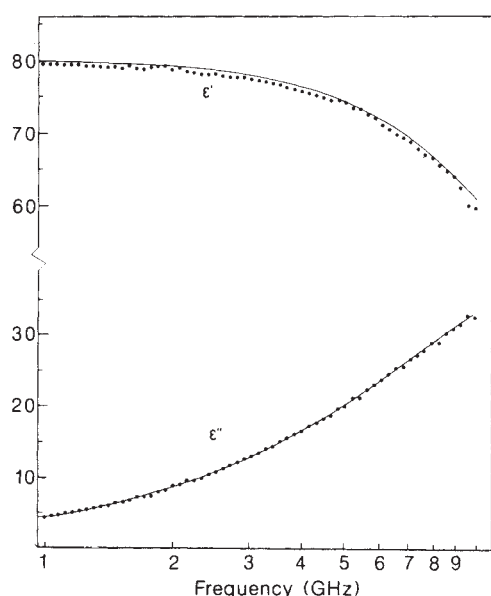


Fig. 2 Relative permittivity (ϵ') and loss factor (ϵ'') of 0.1% plasmid DNA solution at 20 °C. (The full lines are the literature values of the dielectric parameters of pure water.)

slight impedance mismatch within the systems. The technique is therefore particularly suitable for the measurement of dilute aqueous solutions when water is used as reference. The response waveforms of the sample and reference are measured and Fourier transformed, after which the ratio or difference of the transform is used to calculate the relative permittivity (ϵ') and loss factor (ϵ'') of the sample from the appropriate transfer function equation.

The results obtained at King's College are shown in Fig. 1 where it is noticed that a linear scale has been chosen for frequency. This is in order to give a direct comparison with the results of Edwards *et al.*³ who displayed their data in a similar form. The results obtained at Uppsala are shown in Fig. 2. In this case the more traditional logarithmic representation of the frequency has been adopted. The values for the loss factor (ϵ'') in Figs 1 and 2 have been corrected for ionic conductivity, as is customary in this kind of work¹¹. For the sake of comparison

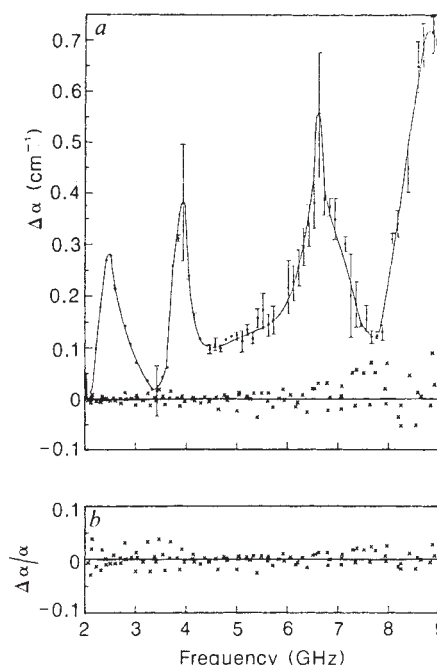


Fig. 3 *a*, Values (α) of incremental attenuation coefficient $\Delta\alpha$ (cm^{-1}) calculated from the data in Figs 1 and 2. The solid line and (●) are data from Edwards *et al.*³ for a 0.053% plasmid DNA solution. *b*, Values of ($\Delta\alpha/\alpha$).

the literature values of the dielectric parameters of pure water are shown as solid curves. In both diagrams each data point is the average of up to 36 independent measurements on samples from four plasmid preparations. Each preparation was characterized by gel electrophoresis before and after the dielectric measurements. The uncertainty in the measured parameters, estimated from measurements on dilute aqueous solutions of known dielectric properties, is of the order of 1% for permittivity and 2% for loss factor. Within these limits the measured dielectric parameters of all samples were close to those of pure water (Figs 1 and 2).

The value of the attenuation coefficient (α) was calculated at each frequency from the measured values of ϵ' and ϵ'' , and the increment ($\Delta\alpha$) over pure water obtained. The variation of $\Delta\alpha$ with frequency is shown in Fig. 3*a* where it is noticed that there is no evidence of resonant behaviour. This is in contrast with the results of Edwards *et al.*³, which are reproduced for the purposes of comparison. The normalized increments ($\Delta\alpha/\alpha$) are shown in Fig. 3*b* where again no deviation from monotonic behaviour outside random scatter is exhibited. Our data thus show that, for a dilute aqueous solution of pUC8.c2 plasmid DNA, in the frequency range 1–10 GHz, no enhanced absorption, resonant or distributed, can be observed.

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1. Maddox, J. *Nature* **324**, 11 (1986).
2. Van Zandt, L. L. *Phys. Rev. Lett.* **57**, 2085–2087 (1986).
3. Edwards, G. C., Davis, C. C., Saffer, J. D. & Swicord, M. L. *Phys. Rev. Lett.* **53**, 1284–1287 (1984).
4. Mie, W. N., Kohli, M., Prohofsky, E. W. & Van Zandt, L. L. *Biopolymers* **20**, 833–852 (1981).
5. Dorfman, B. H. & Van Zandt, L. L. *Biopolymers* **22**, 2639–2665 (1983).
6. Van Zandt, L. L., Kohli, M. & Prohofsky, E. W. *Biopolymers* **21**, 1465–1468 (1982).
7. Scott, A. C. *Phys. Scripta* **32**, 617–623 (1985).
8. Edwards, G. S. thesis, Univ. Maryland (1985).
9. Dawkins, A. W. J. Sheppard, R. J. & Grant, E. H. *J. Phys.* **E12**, 1091–1099 (1979).
10. Gestblom, B. & Elmgren, H. *Chem. Phys. Lett.* **90**, 412–426 (1982).
11. Grant, E. H., Sheppard, R. J. & South, G. P. *Dielectric Behaviour of Biological Molecules in Solution* (Oxford University Press, 1978).

Biogenic etching of microfractures in amorphous and crystalline silicates

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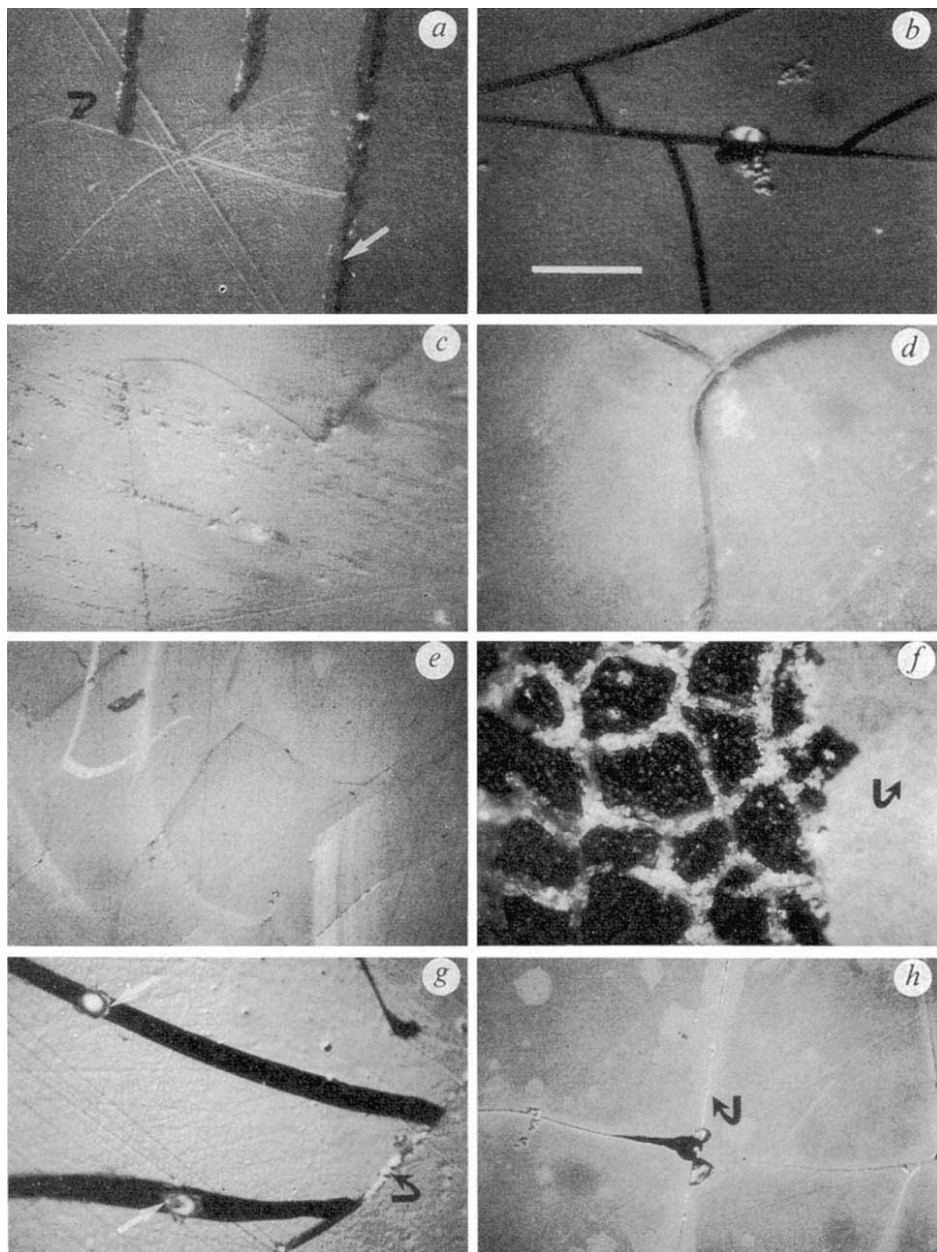
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The weathering of rocks and minerals may involve physical, chemical or biological processes. In particular, as the work of Ehrlich^{1,2}, Aristovska³, Webbly⁴ and their collaborators shows, biogenic weathering in cold and dry environments has generally been attributed to organic acid excretions from living microorganisms^{5,6}. Here we report measurements of the etch rate of microfractures in amorphous and crystalline silicates exposed to either sterilized hot solutions or microcolonies of siderofungi. The biogenic corrosion of the fungi is much faster than that expected

from organic-acid excretions alone, and is probably related to the synthesis of very efficient 'siderophores' (ferric-ion-specific ligands⁷) in the cell membranes, as have already been documented by cell physiologists⁸⁻¹¹ in interpreting the fast transport of iron in cells. This process may act in the natural environment, to produce the differential weathering observed in cosmic spherules made of microscopic 'bars' of olivine and glass, found in terrestrial sediments. These results are relevant to problems ranging from the evolution of soils to the disposal of nuclear wastes.

We first investigated the 'chemical' etching of microfractures in samples of olivine and glasses, exposed to sterilized etchants with no biogenic activity, including hot (100 °C) solutions of distilled water, sea water and citric acid (at a dilution of 10⁻³ M that simulates acid excretions from living microorganisms⁵), as well as much stronger etchants (HF and NaOH). The slabs were cut from the following samples: a single crystal of olivine (MgFeSiO₄) containing about 10 wt % of Fe; a chunk of the most recent borosilicate glass (R7T7) developed in France for the storage of high-level radioactive wastes, and which contains about 30 chemical elements (SiO₂ = 45.5%; Al₂O₃ = 4.9%; B₂O₃ = 14%; Na₂O = 9.9%; CaO = 4%; Fe₂O₃ = 2.9%; ZnO =

Fig. 1 Nomarsky phase contrast images of etched microfractures in olivine and glass R7T7. The set of two images reported on each 'line' of Fig. 1 refers to olivine (on the left) and glass R7T7 (on the right) simultaneously etched in a given etchant. All micrographs have been taken at the same magnification (scale bar *b*, 50 µm), except on line 3 where magnification is 2.5 times smaller. The 4 lines from top to bottom illustrate the effects of a dilute solution of citric acid (2 weeks at 100 °C), sea water (4 weeks at 100 °C), distilled water (3 weeks at 100 °C), and microcolonies of *P. notatum* (3 weeks at room temperature), respectively. For observing microfractures in the glasses we had generally to scrape away (see (*d*) and (*f*)) an opaque reaction layer that coats their external surfaces. In (*f*) we report the boundary between the scraped surface showing the extensive etching of the microfractures (see the whitish linings), and the thick and opaque reaction layer appearing on the right hand side of the micrograph (see dark arrow), and which does not protect microfractures against a deep etching. Much thinner 'transparent' reaction layers were also frequently present on the surface of olivine (look at the attempts for scraping them away in (*c*) and (*e*)). The etching of microfractures in olivine is highly anisotropic, and low etch rates are found along specific crystallographic orientations (see dark arrows in (*a*) and (*g*)); in citric acid, HF and NaOH this corrosion also extends to much shallower depths (see the white arrow in (*a*) identifying the bottom of shallow etch pits produced by citric acid along a microfracture). Our etch rate measurements scale the rate of enlargement of the fractures on the external surface of the slab, which is different from their 'bulk' etch rate, that also depends on the etched depth. Thus they underestimate the very deep biogenic etching of microfractures in olivine by *P. notatum*, relative to citric acid etching, yielding much shallower etch pits.



2.5%; $\text{ZrO}_2 = 1\%$; $\text{Li}_2\text{O} = 2\%$; $\text{P}_2\text{O}_5 = 0.28\%$; and various transition and heavy element oxides = 13%); a soda-lime glass ($\text{SiO}_2 = 75\%$; $\text{Al}_2\text{O}_3 = 1\%$; $\text{Na}_2\text{O} = 11\%$; $\text{CaO} = 9\%$; $\text{MgO} = 2\%$) and an ultra-pure variety of fused silica. Before etching the slabs were heated to 500°C for 15 min and quenched in a bucket of distilled water at 25°C , for the purpose of inducing microfractures by thermal shock. The etch rate of the fractures was subsequently measured with a scanning electron microscope (SEM) and/or an optical microscope, thus enabling the materials to be ranked on a scale of 'durability' against corrosion.

In Fig. 1 we illustrate the following general conclusions of our dissolution tests in sterilized hot solutions for olivine and glass R7T7: the glasses are always much more heavily and deeply corroded than olivine in sea water (Fig. 1c and d), distilled water (Fig. 1e and f), and the HF and NaOH solutions. For citric acid the width of the fractures on the exposed surfaces looks similar in both materials (compare Fig. 1a and b); however their etched depths is again much larger in the glasses. In particular we could not detect any alteration of microfractures in olivine exposed to sea water and distilled water, and from SEM observations we deduced that the fracture etch rates in the soda-lime and R7T7 glasses exposed to the same waters are ≥ 50 times higher than in olivine.

Next we investigated the 'biogenic' etching of microfractures in the same set of solids, resulting from their exposure to microcolonies of siderobacteria and siderofungi (details about the growth procedure are given in Fig. 2). Here we report on our preliminary results obtained so far only on two varieties of fungi known to be surviving in very harsh and cold environments: *Penicillium notatum* was grown on the prefractured slabs, while *Aspergillus amstelodami* was grown on $100\text{-}\mu\text{m}$ -size spheres of a pure variety of fused silica.

The major effects of *P. notatum* on the prefractured slabs are: (1) olivine: no reaction layer was detected, and we could easily observe microfractures in this mineral, deducing that they are clearly etched out at a much faster rate and at much greater depths than in the glasses (compare Fig. 1g and h relevant to olivine and glass R7T7, respectively). Moreover in the widest-opened fractures we could detect spores (identified by relying on criteria given by Prevost¹²), such as those marked by white arrows in Fig. 1g. Such spores, observed up to depths of $50\text{ }\mu\text{m}$, probably act to open further the microfracture network by means of some kind of powerful 'hydraulic' pressure that they generate during growth. (2) Glass R7T7: biogenic etching generates a thin but opaque reaction layer. As this layer is not formed in narrow zones with widths of $20\text{ }\mu\text{m}$ centred along the microfractures (see dark arrow in Fig. 1h), we could observe them with the SEM, noting that their etch rate was at least 20 times lower than the corresponding values measured for olivine. (3) Soda-lime glass: we observed a very friable reaction layer at a few isolated spots on the surface showing a spectacular 'corrosion-print' of the filament morphology of the fungi. Although this reaction layer looks somewhat thicker than that observed on glass R7T7, the fractures in the soda-lime glass were barely etched out. (4) Fused silica: this glass could not be fractured by thermal shock, and we only found evidence for an ultra-thin sticky reaction layer on its surface.

However this last observation, which would imply a strong resistance of fused silica to *P. notatum*, is not applicable to *A. amstelodami*, which present the peculiarity of generating its energy by metabolizing phosphorus¹. In fact we purposely removed phosphorus from the gelose-mineral salt solution to check whether this variety of fungus could quickly adapt its enzymic apparatus for metabolizing silica, thus following earlier suggestions¹. As described in further detail elsewhere¹³, we indeed found that the silica spheres were etched at room temperature by *A. amstelodami*, at a rate at least 100 times faster than the very low rate of dissolution generally quoted for water¹⁴.

It could be argued that this biogenic etching is of a classical type, being related to non-enzymic processes triggered by the

All slabs were sterilized in alcohol and subsequently handled in sterile conditions in a special dust-free hood for avoiding the proliferation of unwanted contaminant microorganisms. For each material two samples were placed in two distinct Petri dishes in a gelose-mineral salt solution containing all usual nutrients except phosphorus. One of the two samples was then sporulated with spores of pure varieties of fungi bought from Laboratoire de Cryptogamie, Muséum d'Histoire Naturelle, Paris, and the other one was used as a blank.

↓

The Petri dishes were then hermetically sealed and stored in the sterile hood at 25°C . After one week well-developed colonies were observed on all samples except the blanks. After two weeks the dishes were briefly opened up, and the pH of the gelose solutions (initial value = 6) were measured in close contact to the colonies; no measurable variation of pH was detected for the *P. notatum* coated slabs but a clear decrease of pH down to values of 4–5 was detected for the silica spheres exposed to *A. amstelodami*

↓

After three weeks the samples were desiccated for about 24 hours under a circulation of dry air in the sterile hood, and the gelose solutions were quickly dissolved in warm (50°C) alcohol

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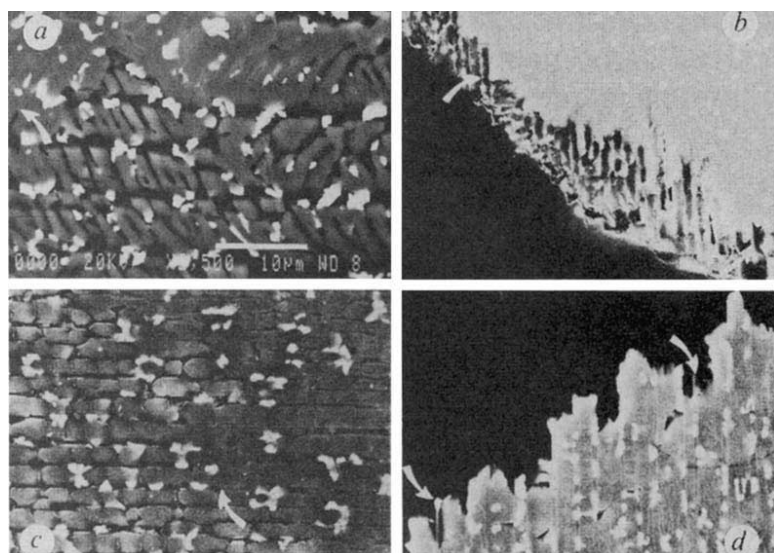
For all samples (including those corroded in the other etchants) the characteristics of the fracture network, such as the width of the fractures and the number of their intersections with a set of circular grids of increasing radius (this last measurement yields the 'fractal' dimension²¹ of the fracture network) were then investigated. The measurements were made with either the SEM or the Nomarsky phase contrast attachment of an optical microscope both before and after the corrosion tests, along specific locations on the polished surfaces identified by means of diamond microscratches. No noticeable corrosion could be detected in the blanks exposed only to the gelose solution; for the unfractured silica spheres exposed to *A. amstelodami*, we just searched with the SEM for various etch features, such as superficial pits and the flattening of the spheres at contact zones

Fig. 2 Synoptic diagram of biogenic etching tests.

end products of all metabolisms, such as excretions of organic acids, which are still quoted in recent discussions⁶. In particular Robert *et al.*⁵ reported for silicates that the group of the 'strongest' organic acids, such as citric acid at the dilution levels (10^{-3} – 10^{-4}M) used in our work, produce an order of magnitude increase in the dissolution rates with regard to values measured for distilled water, but they did not investigate the corrosion of glasses. We believe that acid excretions were not very effective in our biogenic etching tests of olivine. First we exposed for four weeks, at room temperature, a set of prefractured slabs of olivine and glasses in a 10^{-3}M solution of citric acid at 25°C , and we could not detect any measurable alteration of the microfractures in the samples. Next we accelerated this test by heating the same solution of citric acid at 100°C , and after two weeks the etching of microfractures was still much deeper in glass than in olivine (compare Fig. 2c and d). Both tests run opposite to our observations of the very fast and deep etching of microfractures in olivine occurring in about three weeks of exposure to microcolonies of fungus at room temperature, and that corresponds to an enormous increase ($>10^3$) of the fracture etch rate compared to the values deduced for acid excretions either from the data of Robert *et al.*⁵ or from our own dissolution tests in citric acid.

Thus, microorganisms known to adapt quickly to very harsh terrestrial environments, trigger a biogenic etching of microfractures in crystalline and amorphous silicates which cannot be predicted from accelerated dissolution tests conducted on the same materials in hot solutions with no biogenic activity, that cover a broad range of pH (1–10). This etching probably results from the synthesis of siderophores in the cell membranes, which

Fig. 3 SEM images (courtesy of M. Wheelock and C. Jéhanno) of polished sections of four 'barred' chondritic cosmic spherules. In these micrographs, all taken at a similar magnification (scale bar, 10 μm), and all showing a boundary between the etched and non-etched part of a spherule, the three major constituent phases of the spherules in decreasing order of brightness are tiny crystals of magnetite and microscopic bars of olivine and glass; the darker areas are epoxy, filling up the etch canals that penetrate up to depths of $\geq 100\ \mu\text{m}$ and $\leq 10\ \mu\text{m}$ in the deep sea and the Greenland spherules, respectively. In the two spherules extracted from deep-sea sediments, (a) and (c), the microscopic lamellae of interstitial glass (see white arrows), which can also be identified by the presence of tiny magnetite crystals (brightest spots) embedded in the glass, are preferentially etched out. In contrast, in the two Greenland spherules (b) and (d) glass is now the most resistant phase, and the ultra thin network of residual interstitial glass surrounds holes in the microstructure of the Greenland spherules, which result from the complete dissolution of olivine crystals (see the white arrows in (b) and (d) identifying the glass bars). As deep-sea spherules are much more heavily corroded than Greenland spherules their non-etched core can only be observed far away from their edges, which are thus not visible in (a) and (c). By roughly measuring on the SEM images the amount of material removed from the constituent 'bars' of the spherules we deduce that the ratio of the weathering rates of olivine and glass in the Pacific clays and cocoons of Greenland siderobacteria is ≤ 0.05 and ≥ 20 , respectively. Such a wide range of values suggests that the barred cosmic spherules might be useful natural probes, for assessing the corrosive 'power' of their host sediments for silicates, and detecting the onset of biogenic etching which is expected to preferentially etch out olivine.



has already been documented in the literature⁸⁻¹¹ for *P. notatum*. Thus they could etch out olivine at a faster rate than the glasses investigated in this work, as a result of higher concentrations of iron. This preferential etching could be even further 'synergistically' enhanced by the adaptation of their enzymic apparatus for metabolizing the high concentrations of magnesium locked up in olivine, and which is also considered as an important cell nutrient. This process would be less effective in the simulated wastewater glass, which contains a smaller amount of iron (2%) and no magnesium, but also much higher contents of potentially toxic elements (Ag, Al, Zr). It should not operate in the two other types of glasses showing only trace concentrations of Fe.

Finally we investigated the natural weathering of the 'barred' stony cosmic spherules found in terrestrial sediments, and that fortunately happen to be made of microscopic 'bars' of olivine and interstitial glass. In spherules extracted from Pacific deep sea clays, in contact with seawater interstitial solutions, glass is lost at a much faster rate than olivine, in producing a typical pattern of tiny etch canals extending up to depths of 100 μm (Fig. 3a and c). In sharp contrast, in spherules from outwash sediments accumulated in ponds of high purity melt water at 0 °C on the west Greenland ice cap, we observed that olivine is now etched out at faster rate than glass (see Fig. 3b and d). A first clue about this striking difference was obtained when we identified the nature of the Greenland sediments, appearing as mm-size cocoons of microscopic filaments. Standard tests first proposed by Prevost¹² and then modified by Bergey¹⁵, as well as our own determination of an iron-rich shell around the filaments, revealed that they are in fact siderobacteria (Prevost's classification) or 'sheathed' bacteria (Bergey's classification). It might be that their cell membranes also synthesize siderophores, thus initiating a powerful biogenic corrosion somewhat similar to that observed for siderofungi in our laboratory tests. Indeed olivine in the barred spherules show iron-rich rims (up to 30 wt % of Fe), compared to the interstitial Fe-poor glass in which all iron has been reassembled in tiny magnetite crystals, 'shielded' within the glass.

This powerful biogenic process has implications for problems involving the specific area ($\text{m}^2\ \text{g}^{-1}$) of the constituent grains of soils, rocks and man-made structures, which can be considerably increased by the selective opening and/or etching of their microfracture network, and such as: the formation of soils^{1,3}, the

weathering of minerals and rocks¹ (in particular in cold and very dry polar conditions⁶), the related formation of desert varnish¹⁶, the alteration of 'stones' in manmade structure¹⁷, the formation and the mining¹⁸ of mineral ores deposits as assisted by microorganisms and, the circulation of ground waters¹⁹. The present work also gives strong support to the views of West *et al.*²⁰, advocating the importance of studying various effects of biogenic activity on the release of high-level radioactive wastes in the biosphere.

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1. Silverman, M. P. & Ehrlich, H. L. *Adv. appl. Microbiol.* **6**, 153-206 (1964).
2. Ehrlich, H. L. in *Geomicrobiology* (ed. Ehrlich, H. L.) 125-135 (Dekker, New York, 1981).
3. Aristovska, T. V., Daragan, A. Yu., Zykina, L. V. & Kutuzova, R. S. *Soviet Soil Sci.* **5**, 538-546 (1969).
4. Duff, R. B., Webbley, D. M. & Scott, R. O. *Soil Sci.* **95**, 105-114 (1963).
5. Robert, M., Razzaghe, M. A., Vicente, M. A. & Veneau, G. *Science du sol* **2-3**, 153-174 (1978).
6. Ugolini, F. C. in *Rates of Chemical Weathering of Rocks and Minerals* (eds Colman, S. M. & Dethier, D. P.) 193-227 (Academic, Orlando, 1986).
7. Neilands, J. B., Peterson, T. & Leong, S. A. in *Inorganic Chemistry in Biology and Medicine*, American Chemical Society Symp. Ser. 140, 263-278 (1980).
8. Winkelmann, G. in *Proc. NATO Advanced Research Workshop, Iron, Siderophores and Plant Diseases*, East Malling Research Station, Maidstone, Kent, 1-5 July, 1984, Session 17 (ed. Swinburne, T. W.) (Plenum, New York, 1985).
9. De Kock *Proc. NATO Advanced Research Workshop, Iron, Siderophores and Plant Diseases*, East Malling Research Station, Maidstone, Kent, 1-5 July, 1984, Session 17 (ed. Swinburne, T. W.) (Plenum, New York, 1985).
10. Reid, C. P. & Snanizlo, P. J. in *Proc. NATO Advanced Research Workshop, Iron, Siderophores and Plant Diseases*, East Malling Research Station, Maidstone, Kent, 1-5 July, 1984, Session 17 (ed. Swinburn, T. W.) (Plenum, New York, 1985).
11. Schroth, M. N., Loper, J. E. & Yuen, G. Y. in *Proc. NATO Advanced Research Workshop, Iron, Siderophores and Plant Diseases*, East Malling Research Station, Maidstone, Kent, 1-5 July, 1984, Session 13 (ed. Swinburn, T. W.) (Plenum, New York, 1985).
12. Prevost, A. R. in *Traité de Systématique Bactérienne* tome 2 635-657 (Dunod, France, 1961).
13. Pottier, L. thesis, ENSA, Montpellier (1986).
14. Siffert, A. *Mém. Serv. Cont. Géol. Als. Lon.* no. 21 (1962).
15. Bergey, A. in *Manual of Determinative Bacteriology* 8th edn 123 (MacGraw Hill, New York, 1974).
16. Staley, J. T., Palmer, F. & Adams, J. B. *Science* **215**, 1093-1095 (1982).
17. Billy, C. & Blanc, Ph. *Minéraux et Fossiles*, no. 39, 48-52 (1978).
18. Briertley, T. C. *Pour la Science* 38-52 (1982).
19. Matthess, G. in *The Properties of Groundwaters* (Wiley-Interscience, New York, 1982).
20. West, J. M., McKinley, I. G., Grogan, M. A. & Arme, S. C. in *Materials Research Society Symp. Proc. Vol. 50, Meet 1985 on Scientific Basis for Nuclear Waste Management IX* (ed. Werme, L. O.) 533-538 (Materials Research Society, Pittsburgh, 1986).
21. Mandelbrot, B. in *The Fractal Geometry of Nature* (Freeman, New York, 1982).

Changes in the tooth enamel of early Palaeocene mammals allowing increased diet diversity

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Hunter-Schreger bands (HSB) are seen in teeth that are composed of crossed sets of enamel prisms. They are present in the teeth of man and many other mammals¹⁻⁶, but absent in most insectivores and multituberculates⁷⁻⁹. It has been suggested that the presence of HSB makes the tooth enamel less likely to split and is associated with chewing ability¹⁰. We have traced the occurrence of HSB back to the arctonic condylarths of the early Palaeocene (Puercan) age; this must be close to the first appearance of the bands in placental mammals. Our data indicate that the teeth of almost all large mammals since the early Palaeocene have contained these bands, in an orientation that is optimal for limiting the propagation of vertical fractures. The appearance of the bands is associated with the differentiation of herbivores and carnivores from insectivores

and our data indicate that their development was critical to the diversification of mammals because it allowed the use of new types of foods.

The origin and early history of HSB have been unknown. Bands were reported in the enamel of the late Triassic mammal *Morganucodon*⁴, but the structure does not include prism decussation and therefore does not represent HSB. In the early Eocene horse, *Hyracotherium*⁵, however, HSB were found, suggesting that decussation may have been present in the late Palaeocene phenacodontid condylarths. The 'braided prisms', Brauer⁶ referred to in a late Palaeocene actocyonid condylarth from Walbeck, Germany are true HSB.

We found horizontal HSB in molars of *Arctocyon primaevus*, *A. matthesi* (Fig. 1e), *Arctocyonides weigelti* (Cernay les Reims, France; Walbeck, Germany), and in the middle Palaeocene phenacodontid condylarth *Tetraclaenodon*, which is close to the ancestry of the perissodactyls. We then looked in the early Palaeocene (Puercan) mammals from the lower part of the Nacimiento formation of the San Juan basin, New Mexico. We found no HSB in the teeth of the abundant peripitychids (Fig. 1a) of this fauna. But molars of the following arctocyonids proved to have HSB: *Eoconodon heilpranus*, *E. gaudrianus*, *Loxolophus hyattianus*, *Oxycelaenus cf. cuspidatus* and an undetermined arctocyonid.

The HSB appear more poorly developed in these forms (Fig. 1b, c, d) than in the late Palaeocene representatives of *Arctocyon* (Fig. 1e, f). Conspicuous decussation occurs only in the centre of the enamel with smaller angles (Fig. 1d) suggesting primitive conditions.

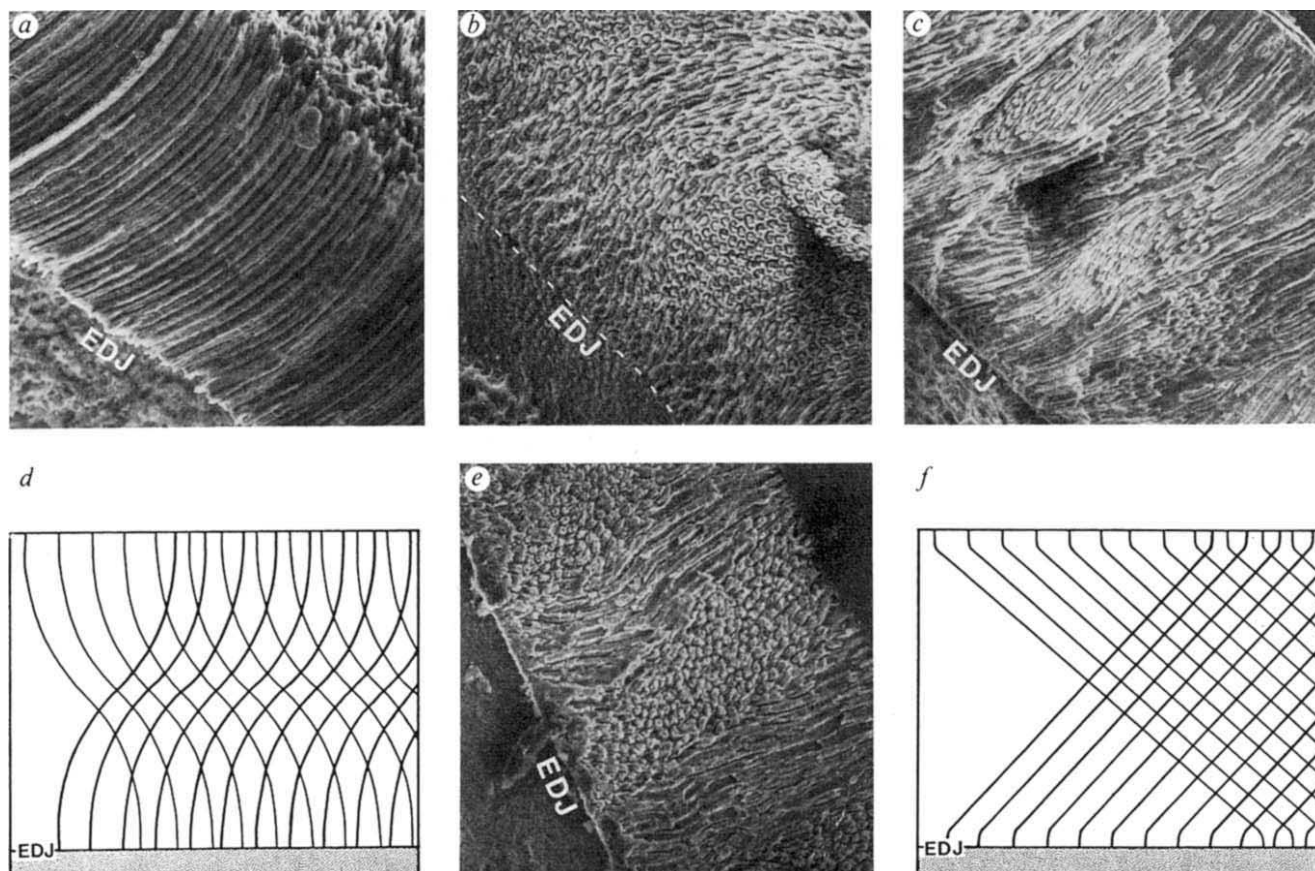


Fig. 1 Enamel in Palaeocene mammals. SEM micrographs of vertical sections and reconstructions of the course of prisms in a horizontal plane parallel to prisms. EDJ, enamel-dentine junction. a, Early Palaeocene condylarth *Conacodon* (San Juan basin, New Mexico) with completely parallel prisms (no HSB) and fracture surface following prism borders; b, c, early Palaeocene arctocyonid (San Juan basin) with Hunter-Schreger bands (HSB) limited to central part of enamel, lower angle decussation; d, schematic reconstruction of HSB in early Palaeocene arctocyonid enamel; e, late Palaeocene *Arctocyon matthesi* (Walbeck, Germany) with distinct HSB extending through entire thickness and nearly at 90° decussation; f, schematic reconstruction of HSB in late Palaeocene arctocyonid enamel. a, c, Broken sections (not ground or polished); b, e, polished sections; all sections were etched slightly with HCl.

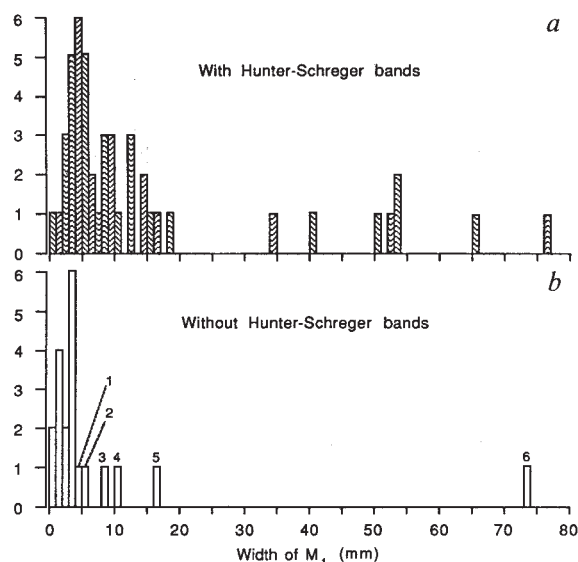


Fig. 2 Frequency distributions of size (as width of M_1) in eutherian genera *a*, with and *b*, without HSB. Data are for all genera in which the presence or absence of HSB has been published or observed and for which we have measurements of the size of M_1 . Larger mammals lacking HSB: 1, *Conacodon* (peripitychid condylarth); 2, *Alouatta* (cebid primate); 3, *Globicephala* (delphinid cetacean); 4, *Carsiptychus* (peripitychid condylarth); 5, *Halitherium* (dugonid sirenian); 6, *Physeter* (physeterid cetacean). Statistical tests noted in the text included in addition to these genera *Orcinus* (delphinid cetacean) and *Berardius* (ziphiid cetacean), which fall in the category of large forms lacking HSB.

We found no prism decussation in the oldest known arctocyonid, *Protungulatum donnae*, which occurs in the late Cretaceous (or possibly earliest Palaeocene) Bug Creek fauna of Montana, nor in other late Mesozoic mammals we examined (*Climolestes*, *Procerberus*). Because the arctocyonids are the only early Palaeocene mammals found to have HSB and the bands are poorly developed, it appears that the early palaeocene marks the first appearance of HSB. There is no other lineage, including Multituberculata⁷⁻⁹ of Mesozoic or early Palaeocene mammals in which HSB have been found.

The timing of this early occurrence of HSB coincides with the emerging radiation of mammals of increasing size after the disappearance of dinosaur-dominated Cretaceous faunas. Among the studied Cenozoic therian mammals, there appears to be an association between large body size and HSB. To assess the degree of this association, we collected measurements of the width of the first lower molar in as many mammals as available in which the presence or absence of HSB is known (Fig. 2). Among the eutherians, HSB are generally absent in insectivora and chiroptera¹, but appear to be present in Carnivora and ungulates^{2,5,11}. In Primates, they are present in most taxa with large body size, but are absent in all examined small Eocene prosimians. The occurrence of HSB in larger marsupials^{1,12} parallels that in the eutherians.

The probability that there is no association between the presence of HSB and molar width of 4 mm or greater in the data of Fig. 3 is low (<0.002). We also tested a smaller subset consisting of one representative of each family unless a family varied in the presence/absence of HSB or in molar width above and below 4 mm (null probability >0.01). These data are of course a small sample of all mammals.

Exceptions to the association between body size and HSB are expected because of the stochastic nature of mutation and the possibility of large animals preferring soft diet (for example, fish-eaters). The exceptions are few, however. HSB are present in the archeocete whales and in their presumed ancestors

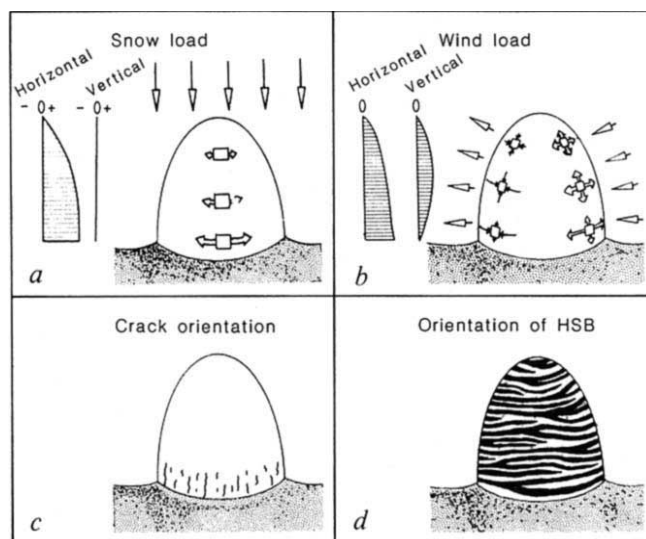


Fig. 3 Shell model of compressive and tensile vectors in enamel. *a*, effects of a vertical 'snow' load impinging on all parts of the crown; *b*, effects of horizontal or 'wind' load; *c*, crack orientation and location commonly observed in mammalian teeth and which is predicted by *a* and *b*; *d*, typical orientation of HSB in low crowned cusps and conical teeth of therian mammals.

(mesonychids)¹³ but are absent in later toothed Cetacea^{1,2}, due to a reduction or loss of enamel.

Furthermore only two groups of mammals containing members with first molars larger than 6 mm are known to lack HSB: Sirenia, that may have derived from phenacodontids, that have HSB, and all examined peripitychids (Fig. 1*a*), which were largely replaced by the phenacodontids and arctocyonids, groups with HSB.

The only major exception to the general absence of HSB in small mammals are the rodents^{2,14}. Among the insectivora, we found HSB only in the incisors of *Erinaceus*. HSB are present in the European carnivora *Mustela nivalis* and the ceboid primate *Callithrix*, but these diminutive species presumably inherited their HSB from larger ancestors.

The prominent development of HSB in the incisors of rodents and the differing or poorer development of HSB in rodent cheek teeth¹⁴ suggests a relationship between HSB and chewing stress, because gnawing exerts high stresses. The appearance of HSB across the Cretaceous-Tertiary boundary in mammals of larger size may be due to increased chewing stress.

Chewing pressure varies directly with the cross-sectional area of masticatory muscles and inversely with the area of the chewing surface. An increase in body size would not produce increased average chewing pressure if the proportions are retained. However, in any case in which there is a general size increase the maximum chewing force increases, and small, resistant and stress-concentrating objects elevate the stress to a much higher level than is possible in smaller mammals.

Furthermore, in conjunction with increasing size, feeding habits shifted from soft insects to herbivorous or carnivorous diets requiring higher chewing pressures.

HSB arose as horizontal layers of decussating prisms. Enamel, as a compound material, is less likely to fail by compression than by tension. The tensile strength of enamel has been measured to range from 10.3 to 65 MNm⁻² and the compressive strength to range from 94.5 to 385 MNm⁻² (ref. 15). A shell

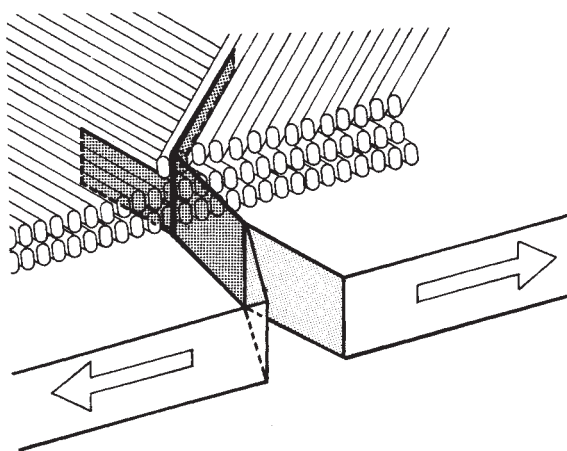


Fig. 4 Relationship between prism direction and crack propagation in HSB. The tendency for horizontal tensional force to produce vertical cracks which, in enamel reinforced by Hunter-Schreger bands (HSB), tend to divide owing to the different prism directions in overlying and underlying bands.

theory model¹⁶⁻¹⁹ applied to a simple unicuspid tooth or tooth element shows horizontal tension forces to occur under occlusal load ('snow-load', Fig. 3a) as well as under bending load ('wind-load', Fig. 3b). Those tension forces will cause vertical cracks. Indeed small vertical cracks can be found at the base of the enamel, where the calculated tension forces reach a maximum (Fig. 3c). Prism decussation strengthens the enamel in respect to the horizontal tension forces like plywood (Fig. 3d). Propagation of vertical cracks is also restrained. The cracks run parallel to the prisms and choose different directions in adjacent HSB leaving a typical Y-shaped pattern, which can easily be observed in artificial cracks (Fig. 4). The branches of these cracks are inclined to the tension forces and therefore soon stop.

The polyphyletic appearance of the HSB during the early Cenozoic, dominance in large forms, and appearance in small forms with specialized mechanisms that increase dental stress, imply that enamel strength thresholds were critical in the transitions to Cenozoic herbivores and carnivores. The early innovation of HSB occurred in mammals in which the occlusal mechanisms had not changed greatly, suggesting that the change in scale was the most important factor.

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1. Kawai, N. *Okijamas Fol. Anat. Jap.* **27**, 115-131 (1955).
2. Beier, K. *Zool. Anz.* **207**, 288-299 (1981).
3. Tomes, J. *Phil. Trans. R. Soc. Lond.* **1850**, 529-567 (1850).
4. Grine F. E. & Cruickshank, A. R. I. *Proc. Electron Microscopy Sci. S. Africa* **8**, 121-122 (1978).
5. Rensberger, J. M. & Koenigswald, W. v. *Paleobiology* **6**, 447-495 (1980).
6. Brauer, R. *Nova Acta Leopold. NF. Halle* **13**, 61-127 (1943).
7. Sahni, A. *Palaeontographica* **166**, 37-49 (1979).
8. Fosse, G., Kielan-Jaworowska, Z. & Skaale, S. G. *Paleontology* **28**, 435-449 (1985).
9. Carlson, S. J. & Krause, D. W. *Contr. Mus. Paleont. Univ. Michigan* **27**, 1-50 (1985).
10. Koenigswald, W. v. *Abh. senckenberg. naturforsch. Ges. Frankfurt* **539**, 1-129 (1980).
11. Fortelius, M. *Acta Zool. Fennica* **180**, 1-76 (1985).
12. Beier, C. *Zool. Anz. Jena* **210**, 315-332 (1983).
13. Sahni, A. *J. Paleont. Soc. India* **25**, 33-37 (1981).
14. Korvenkontio, V. A. *Ann. Zool. Soc. Zool.-Bot., Fennica Vanamo* **2**, 1-274 (1934).
15. Waters, N. E. *Symp. Soc. Experiment. Biol.* **34**, 99-135 (1980).
16. Flüge, W. *Statik und Dynamik in Schalen* (Springer, Berlin, 1981).
17. Powers, J. M., Craig, R. G. & Ludema, K. C. *J. Dental Res.* **52**, 1327-1331 (1973).
18. Boyde, A. *Operative Dent.* **1**, 13-28 (1976).
19. Hassan, R., Caputo, A. A. & Bunshah, R. F. *J. Dental Res.* **60**, 820-827 (1981).

Morphogenesis of a tiered principal retina and the evolution of jumping spiders

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The principal eyes of jumping spiders (Salticidae) possess a unique visual apparatus¹ of unknown phylogenetic origin. Their retinas, with four axial tiers of receptive segments, lie at the end of long, motile tubes² and have the transverse profiles of narrow, vertical 'boomerangs' with very small horizontal acceptance angles. No other ocellar retina resembles them, and no evolutionary pathway for the transition between the simple, hemispherical monolayers of receptors found elsewhere and the sophisticated jumping spider eye has been proposed. We show that the post-embryonic morphogenesis of a principal retina may explain how it evolved. Conformational changes generate the narrow retinal mosaics from a hemisphere of presumptive receptive segments with hexagonal packing and irregular microvilli distributed around their perimeters much as they are in the retinas of more primitive spiders³. Tiering is a consequence of the conformational change. Our explanation has significant implications for the evolution of the Salticidae.

The principal retinas of jumping spiders and their mode of operation² are an elaboration of the arthropod ocellus. They can sustain spatial acuities at the fovea of as little as 2.4 arc min and are supplied with images by a telephoto lens system^{4,5}. Of the four axial tiers of receptive segments, layer I, farthest from the dioptrics, consists of a high-acuity mosaic composed of long, single rhabdomeres organized as light-guides, the more distal rhabdomeres of layers II-IV being short, and precluded from light-guiding by associated mitochondria that equilibrate their refractive indices to those of their surrounds^{4,5}.

How did such a complex and, in terms of the behaviour of the spiders⁶, efficient system evolve? Early stages in the evolution of the principal retina are not conserved by any contemporary spider. However, a primitive sub-family of the Salticidae, the *Spartaeinae*⁷ includes *Yaginumanis*⁸, in which layer-I receptive segments each contain two rhabdomeres so disposed as to generate optical pooling between contiguous receptors; *Spartaeus*⁹, with a transitional retina; and *Portia*, in which the remarkable spatial acuity indicated by ethological experiments⁶ is sustained by optimization of the foveal layer-I mosaic so that its rhabdomeres act as light guides^{4,10}, just as they do in advanced Salticids, including *Plexippus*^{5,11}. Within the Salticidae there is no evidence as to how retinal tiering arose during phylogeny or is achieved during development. An early embryological study¹² described the formation of the mature retinal strip, but failed to recognize that the retina is tiered.

The post-embryonic morphogenesis of the principal retina provides a clue to the evolutionary pathway that may have led to it, given some element of recapitulation. Despite some obvious reservations, natural selection acts upon morphogenetic cascades. A contemporary developmental sequence can indicate what may have happened during phylogeny¹³.

Post-embryos of *Plexippus validus* (Urquhart) were held at 20±1°C and sampled from the second day after emergence from the chorion (day 2) until three days after moulting to the second instar at day 12, using standard techniques of fixation for electron microscopy⁹. Second-instar spiderlings leave their brood sacs and become independent at day 15. Changes to the gross morphology of the retina were studied from serial semi-thin resin sections stained with toluidine blue. Criteria for recogniz-

Fig. 1 *a*, Schematic longitudinal profile of a principal eye of an adult Salticid. The retina, with four (1–4) tiers of foveal receptors lies at the proximal end of a long, effectively fluid-filled tube. A corneal lens provides images to the retina which are magnified by a diverging component, the Pit⁴. *b*, Orientation of *c–f* *in vivo*. D, dorsal; V, ventral; L, lateral; M, medial. *c–f*, Diagram of the conformational changes undergone by a retina during early post-embryonic development, derived from semi-thin resin sections. *c*, Day 3: the retina is a simple hemisphere. Progressively, it narrows with respect to the horizontal axis (*h*). *d*, Days 4–5: narrowing commences at the ventral margin. *e*, Day 7: narrowing now involves the dorsal margin of the retina, and the lateral and medial margins have started to generate the narrow foveal region by migration towards each other. *f*, Day 10: the transverse profile of the retina has begun to approximate to the mature 'boomerang' conformation (illustrated for *Plexippus* in ref. 5). Infolding of the developing retina along *h*, and a progressive increase in the lengths of the receptive segments, especially at the fovea, produce both the final tiering of the receptive segments and a marked increase in the overall depth of the retina.

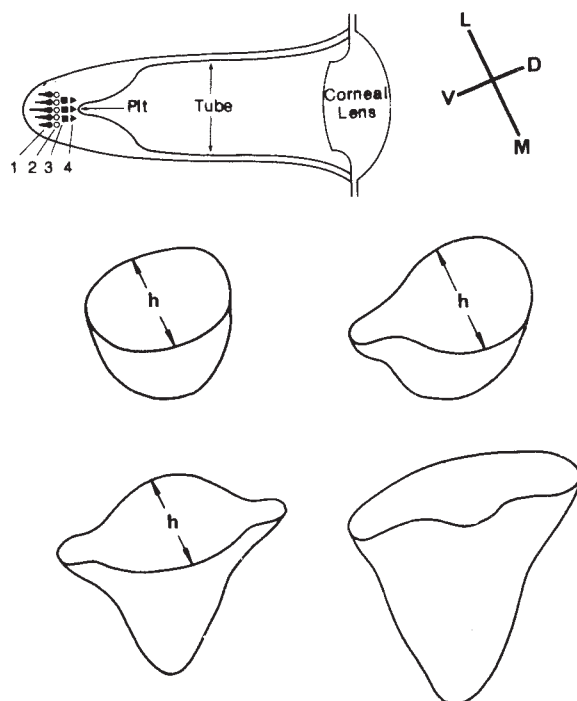
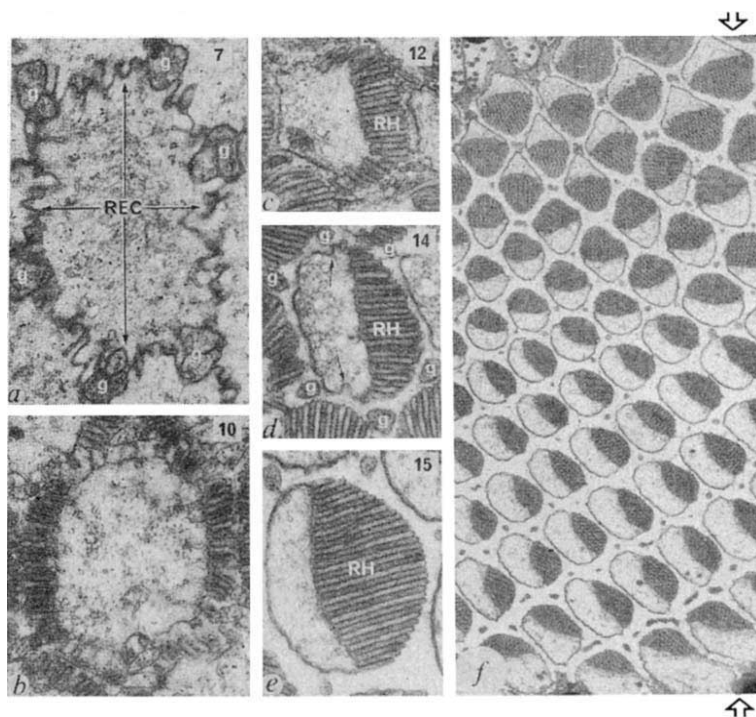


Fig. 2 Transverse sections of foveal or near-foveal receptive segments in layer I. *a*, Day 7: the plasmalemma of a receptive segment (REC) is irregularly pleated, devoid of microvilli and flanked by six longitudinal glial columns (*g*). *b*, Day 10: short, irregular microvilli constitute two faces of the receptive segment. Receptive segments shown in *a* and *b* both lie to one side of the fovea and are larger than those at the optical axis. *c*, Day 12: a developing rhabdomere (RH) constitutes one face of the receptive segment. Adjacent receptive segments are contiguous. *d*, Day 14: a rhabdomere consists of longer, more regular microvilli. The cross-sectional area of the receptive segment is in the process of reduction by vigorous plasmalemmal endocytosis; arrows, endocytotic pits. *e*, Day 15: receptive segments are well-separated, allowing rhabdomeres to act as light guides; the rhabdomere shown is fully-formed, with regular microvilli. *f*, Overview of the foveal receptor mosaic at day 15. The glial strands are greatly reduced in thickness. Magnification: *a–e*, $\times 25,000$; *f*, $\times 7,500$.



ing stages of post-embryonic development in spiders that do not hatch quite synchronously in a batch will be given in full elsewhere¹⁴, together with a detailed account of the conformational changes summarized below.

Figure 1 shows in outline the main events of post-embryonic principal retinal development. By day 3, the presumptive principal retina is established as a hemisphere which presents a receptor mosaic with hexagonal symmetry. At around days 4–5 there is a dramatic conformational change: the retina narrows to form an ellipse with a vertical (that is, dorsoventral) long axis, starting ventrally. Thus, at day 3, the equatorial margin of the future receptive segment field has a horizontal diameter of $\sim 90 \mu\text{m}$ which at the completion of narrowing at day 11 is

reduced to $\sim 20 \mu\text{m}$. Neither mitotic figures nor symptoms of cell death are observed between day 2 and day 15, so that the acquisition of new receptive segments is a consequence solely of cellular differentiation. Changes in the distribution of receptive segments during the course of narrowing¹⁴ imply that segments are concurrently being added at the margins of the cup. There is no evidence that there is any marked differential migration of receptive segments along a line parallel to the optical axis, and foveal layer-I receptive segments, which we postulate to differentiate first, seem to remain at a stable position at the retinal pole, a conclusion that is reinforced by electron microscopy¹⁵. Thus nascent receptive segments at or near to the primordial equator are thought to give rise to the distal layers

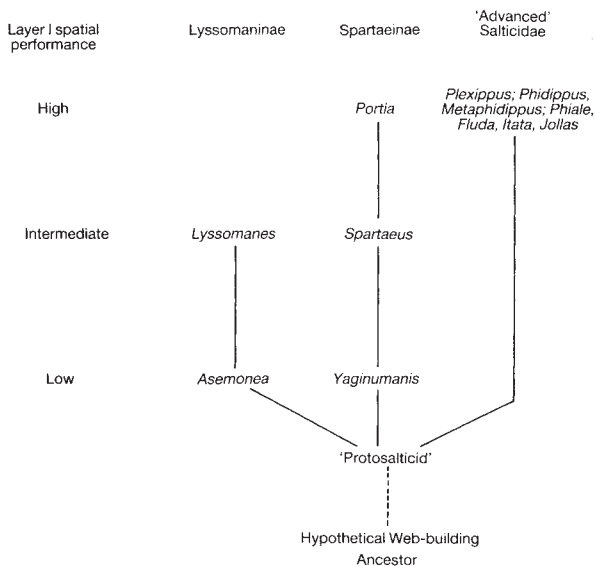


Fig. 3 Some relationships between the salticid genera mentioned in the text. We assume from the topographical homologies between all principal retinas that tiering evolved only once. Thus, a hypothetical web-building ancestor¹⁷ gave rise to a 'protosalticid' with a simple tiered retina, from which the Lyssomaninae, the Spartaeinae and the several families of 'advanced' salticids (for example, the Dendryphantinae^{1,2,22}) were derived as independent lineages. Layer-I receptor mosaics are distinguished as able to support low, intermediate and high spatial acuities, if provided with appropriate dioptrics, in terms of their geometrical regularity and the varying capacity of their rhabdoms to act as light guides. The genera in the dendrogram exemplify stages in the evolution of high-resolution layer-I mosaics and do not represent phylogenetic lineages. The principal retina of *Asemonea* (A.D.B. and M.C., in preparation) functionally resembles that of *Yaginumanis*⁸; its layer-I mosaic is close to the rhabdomeral network that we postulate for a hypothetical protosalticid. References for other species are shown where they occur in the text, except see ref. 22 for *Phidippus* and *Metaphidippus*, and ref. 11 for *Phiale*, *Fluda*, *Itata* and *Jollas*.

IV and III, and narrowing is believed to force them inwards so that they ultimately overlie those of layers I and II. These changes are consistent with the pattern of entry of receptive segment dendrites into the retinal cup: those of layer I enter along lines more-or-less parallel to the optical axis, whereas those of all three distal layers enter more-or-less at right angles to it.

The following ultrastructural account concerns layer I only (although layers II-IV develop in similar ways¹⁵). Initially, receptive segments, however well-defined, do not bear microvilli and lack them as late as day 7. Each receptive segment is flanked by six separate, sub-divided strands derived from the pigmented glia¹⁶ (Fig. 2a). Throughout post-embryonic development, glial strands increase in volume in the peripheral retina to form the matrix in which the mature receptive segments are embedded. At day 10, receptive segments bear irregular microvilli on all faces (Fig. 2b) with the exception of foveal segments where they tend to be present on one face only, anticipating their mature disposition. The predominant arrangement resembles that of the receptive segments in the principal eyes of relatively primitive araneomorph spiders in the Clubionidae, Sparassidae and Gnaphosidae³. Final reorganization of microvilli and of their dispositions in receptive segments to yield the mature pattern occurs after the post-embryos moult to the second instar between day 13 and day 15 (Fig. 2c-f).

The principal eyes of a few, rather distantly related families of araneomorph spiders have been shown to differentiate from an infolding of the embryonic blastoderm which gives rise to

both the 'glass cells' that generate the lens and the components of the retina¹². Early morphogenesis of such retinas leads directly to the formation of near-hemispheres of nascent receptive segments¹² for the lycosid *Alopecosa*. The conformational events that we describe appear to be added onto a developmental sequence that will probably prove common to the retinas of all araneomorph spiders.

The morphogenetic sequence that we have summarized and that will be described in detail elsewhere^{14,15} allows the possibility of a direct phylogenetic transition from the rhabdomeral networks of more primitive spiders³ to the tiered retinas of the Salticidae, given an assumption that the morphogenetic events can be supposed to recapitulate an evolutionary sequence. It has recently been suggested that the Salticidae may not have evolved from a pre-existing group of cursorial spiders, but from web-builders that first acquired the tactic of invading the webs of other families of spiders in order to prey upon their occupants¹⁷. Given that many web-building spiders are known to have poorly developed principal eyes with rhabdomeral networks³, a direct ontogenetic transition is at least consistent with the hypothesis, and certainly offers nothing to contradict it.

A tentative phylogeny for the Salticidae which combines the hypothesis that jumping spiders evolved from web-builders¹⁷ with our current understanding of their principal and secondary eyes is shown in Fig. 3. The separation of the Lyssomaninae and Spartaeinae from a number of sub-families that are notionally advanced is well-established⁷; the inter-relationships of the sub-families of 'advanced forms' is less well understood (F. R. Wanless, personal communication). The dendrogram presented here is not intended to pre-empt relationships inferred by taxonomists from more conventional characters.

Salticid principal retinas are known, reliably, to possess green and ultraviolet receptors^{5,18}, the latter lying distally in layer IV as optical modelling demands because of the chromatic aberration of the dioptrics^{1,4,5}. A study of a Japanese species *Menemerus* found yellow, green, blue and ultraviolet receptors in a sample of seven unmarked cells¹⁹ and inferred that the yellow receptors were in layer I, a conclusion that optical constraints render unlikely⁵. Principal eyes of an araneid in the genus *Argyope* contain green, blue and ultraviolet receptors²⁰, although their rhabdomeral networks cannot sustain accurate image analysis^{3,21}. Generation of a tiered retina by the inwards displacement of initially equatorial receptive segments means that a precursor of the Salticidae may have possessed ultraviolet receptors restricted to the retinal margin. Such an arrangement and its consequences would be consistent with a puzzling feature of the Salticid retinas we have examined: in the primitive *Yaginumanis* and *Lyssomanes* the characteristic topography of outer layer-IV receptive segments is already present, although the optical optimization of foveal layer-I segments has not been achieved⁸. A conserved marginal strip of ultraviolet receptors is an attractive possibility. (For a review of studies suggesting that such a strip is used for navigation by sky polarization in the agelenid genus *Agelena* see ref. 15.)

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1. Land, M. F. *J. exp. Biol.* **51**, 433-470 (1969).
2. Land, M. F. *J. exp. Biol.* **51**, 471-493 (1969).
3. Blest, A. D. *The Neurobiology of Arachnids* (ed. Barth, F. G.) 79-102 (Springer, Berlin, 1985).
4. Williams, D. S. & McIntyre, P. *Nature* **288**, 578-580 (1980).
5. Blest, A. D., Hardie, R. C., McIntyre, P. & Williams, D. S. *J. comp. Physiol. A* **145**, 227-239 (1981).
6. Jackson, R. R. & Blest, A. D. *J. exp. Biol.* **97**, 441-445 (1982).
7. Wanless, F. R. *Bull. Brit. Mus. nat. Hist. Zool.* **46**, 135-205 (1984).
8. Blest, A. D. & Sigmund, C. *Proc. R. Soc. B* **221**, 111-125 (1984).
9. Blest, A. D. & Sigmund, C. *Protoplasma* **125**, 129-139 (1985).
10. Blest, A. D. & Price, G. D. *Protoplasma* **120**, 172-184 (1984).
11. Blest, A. D. *J. comp. Physiol. A* **157**, 391-404 (1985).
12. Homann, H. Z. *Morph. Tiere* **69**, 201-272 (1971).
13. De Beer, G. R. *Embryos and Ancestors* 3rd edn (Oxford University Press, 1958).
14. Blest, A. D. *Phil. Trans. R. Soc. B* (in the press).
15. Blest, A. D. & Carter, M. *Phil. Trans. R. Soc. B* (in the press).

16. Eakin, R. M. & Brandenburger, J. J. *Ultrastruct. Res.* **37**, 618–663 (1971).
17. Jackson, R. R. & Blest, A. D. *J. Zool. Lond.* **196**, 255–293 (1982).
18. De Voe, R. D. *J. gen. Physiol.* **66**, 193–208 (1975).
19. Yamashita, S. & Tateda, H. *J. comp. Physiol. A* **105**, 29–41 (1978).
20. Yamashita, S. & Tateda, H. *J. exp. Biol.* **74**, 47–58 (1978).
21. Uehara, A., Toh, Y. & Tateda, H. *Cell Tiss. Res.* **182**, 81–91 (1977).
22. Blest, A. D., McIntyre, P. & Carter, M. *J. comp. Physiol.* (in the press).

Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve

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Formation of myelinated tracts in central nervous system (CNS) regions such as the optic nerve seems to depend on two glial cell types, both of which derive from a common progenitor cell. This oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell gives rise to oligodendrocytes, which produce internodal myelin sheaths, and to type-2 astrocytes, which extend fine processes in the region of the nodal axolemma^{1,2}. The optic nerve also contains a third glial cell, the type-1 astrocyte, which derives from a separate precursor¹. These three glial cells develop in a fixed sequence over a two-week period³: type-1 astrocytes appear at embryonic day 16 (E16), oligodendrocytes at the day of birth (E21 or postnatal day P0), and type-2 astrocytes between P8 and P10. Type-1 astrocytes secrete a potent mitogen which causes expansion of the O-2A progenitor cell population *in vitro*^{4,5}. Here, we report that dividing O-2A progenitor cells are highly motile and seem to migrate from the brain into the optic nerve, beginning at its chiasmal end. Our results indicate that long-distance migration along the neural axis is characteristic only of progenitors of the O-2A lineage and may serve to distribute these cells to regions of the CNS that will become myelinated. These results also suggest that the intrinsic neuroepithelial cells of the optic stalk may be even more restricted than previously thought, giving rise only to type-1 astrocytes.

To understand how the optic nerve comes to contain two distinct glial lineages, the distribution of O-2A progenitor cells was analysed by dividing perinatal nerves into three equal segments (Table 1). No progenitor-like cells were detected in the retinal portion of E16 nerves (14–15 mm embryos), but small numbers were found towards the chiasmal pole. In the E17 nerve, cells with an O-2A progenitor-like phenotype were distributed in a strikingly graded fashion: such cells represented <0.4% of the cells in suspensions from the retinal segments, 2.4% of the middle segments and 6.5% of the chiasmal segments, whereas in the anterior half of the optic chiasm, they represented 12.2% of the cells. In fact, at E17, the optic chiasm contained almost 10 times more progenitor-like cells than the entire nerve. At birth, a steep gradient was still present: 18% of the total progenitor-like cell population of the nerve occurred in the retinal segment, 31% in the middle segment and 51% in the chiasmal segment. This gradient diminished gradually during the first nine postnatal days.

To confirm that progenitor-like cells analysed in 4-hour cultures were *bona fide* O-2A progenitors, we cultured cells for 3–4 days in either chemically-defined medium or medium containing 10% fetal calf serum (FCS), conditions in which the great majority of progenitor cells differentiate into oligoden-

Table 1 Distribution of O-2A progenitor-like cells in perinatal rat optic nerves

Age	No. of progenitor-like cells per nerve segment (NSP-4 ⁺ , GC ⁺ , GFAP ⁺)*			Total O-2A progenitor- like cells per nerve
	Retinal	Middle	Chiasmal	
E16	0	17 ± 5	89 ± 18	106
E17	9 ± 6	73 ± 12	236 ± 31	318
P0	417 ± 82	728 ± 102	1,194 ± 84	2,339
P5	2,787 ± 106	2,846 ± 91	3,884 ± 129	9,517
P9	4,001 ± 463	5,380 ± 634	5,438 ± 609	14,819

Optic nerves were divided into three equal segments termed retinal, middle and chiasmal based on position. Cells from each segment were dissociated with collagenase, trypsin and EDTA followed by gentle trituration⁴. Optimal enzyme incubation times were determined for each age on the basis of maximum cell yield. Cells were counted in a haemocytometer and then 10,000 cells were plated on poly-L-lysine-coated glass coverslips in L-15 medium without FCS for 3–4 h. Coverslips were prefixed in 2% paraformaldehyde for 10 min to minimize cell loss during labelling. In most experiments cells were double-labelled, first with one of the following two monoclonal antibodies: anti-GC (ref. 16) (ascites fluid, 1:100) or anti-NSP-4 (ref. 17) (culture supernatant, 1:2) followed by rhodamine-conjugated goat anti-mouse immunoglobulin (Cappel, 1:100) and then after fixing in acid-alcohol, cells were incubated in rabbit anti-GFAP serum¹⁸ (1:1,000) followed by fluorescein-conjugated sheep anti-rabbit immunoglobulin (Wellcome, 1:100). For older ages, P0–P9, some coverslips were triple-labelled with anti-GC, followed by fluorescein-conjugated goat anti-IgG₃ (Nordic, 1:50), and then anti-NSP-4, and anti-GFAP as above. Incubation times of 30 min were extended to 60 min when cells were found negative for a label. Results (mean ± s.d.) are the number of cells isolated from each segment multiplied by the proportion of O-2A progenitor cells as assessed by immunofluorescence in three or more experiments. The phenotypes of cultured rat optic nerve glial cells, as previously described^{1,2}, are listed below: O-2A progenitor cells: bipolar, NSP-4⁺, A2B5⁺, GC⁺, GFAP⁺. Oligodendrocytes: multipolar, NSP-4⁺, GC⁺, GFAP⁺, A2B5⁺/. (The specific gangliosides labelled by the A2B5 (ref. 19) monoclonal antibody are initially expressed by oligodendrocytes but then lost as these cells mature.) Type-2 astrocytes: multipolar, NSP-4⁺, A2B5⁺, GC⁺, GFAP⁺; type-1 astrocytes: nonprocess-bearing, NSP-4⁺, A2B5⁺, GC⁺, GFAP⁺.

* Abbreviations: GC, galactocerebroside; GFAP, glial fibrillary acidic protein.

drocytes or type-2 astrocytes, respectively¹. Because oligodendrocytes and type-2 astrocytes do not develop in the nerve *in vivo* until after birth, the development of these cells in cultures of embryonic nerve must reflect the presence of progenitors in the starting population. Oligodendrocytes or type-2 astrocytes developed only in cultures prepared from those regions of E16 and P0 optic nerves where progenitor-like cells were observed in 4-h cultures (Table 2).

One explanation for these results is that maturation of optic stalk cells occurs in a graded fashion proceeding from the chiasmal end towards the eye. Two observations suggest this is not the case. First, no O-2A lineage cells developed in cultures prepared from retinal segments of E16 nerves even after 12 days of growth *in vitro* (Table 2). Co-culture of cells from the retinal and chiasmal segments of E16 nerves (established by means of spacers to separate the two populations) failed to provide evidence for soluble factors in the chiasmal segment capable of inducing O-2A lineage cells from the retinal segment. These results suggest the neuroepithelial cells of the optic stalk may not be able to generate the O-2A cell lineage, just as they seem unable to generate neurons. Second, if differentiation of optic stalk cells proceeds as a general gradient from the chiasm towards the eye, more type-1 astrocytes might be expected in the chiasmal than in the retinal half of the nerve early in development. In contrast, the opposite result was obtained: the first type-1 astrocytes detected at E16 were almost entirely restricted to the retinal half of the nerve (Table 3). Three days

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Table 2 Effect of culture media on the development of O-2A progenitor cells

Nerve segments	E16		P0	
	Type-2 astrocytes*	Oligodendrocytes†	Type-2 astrocytes	Oligodendrocytes
BS+0.5% FCS medium:				
Retinal	0	0±1	0	24±6
Middle	0	5±3	0	59±12
Chiasmal	0	23±8	0	128±15
DMEM+10% FCS medium:				
Retinal	0	0	9±4	0
Middle	8±4	0	36±8	4±2
Chiasmal	34±5	0	80±11	12±2

E16 and P0 optic nerves were divided into three segments and dissociated as in Table 1 legend. Cells (10,000) were plated in 15 µl of L-15 medium in the centre of a poly-L-lysine-coated glass coverslip placed in a Falcon multi-well plate for 30 min after which 500 µl of either of the following media were added: a modified Bottenstein-Sato medium²⁰ with 0.5% (v/v) FCS (B/S+0.5% FCS) described previously⁴ or Dulbecco's modified Eagle's medium containing 10% (v/v) FCS (DMEM+10% FCS). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for three days (P0) or four days (E16) and then triple-labelled with anti-GC, anti-NSP-4 and anti-GFAP as in Table 1. Numbers represent the mean±s.d. calculated from at least three separate experiments. Additional coverslips from the retinal segment of E16 nerves were immunolabelled every 2–3 days, as above, for a culture period of up to 12 days.

* Type-2 astrocytes were defined as NSP-4⁺, GC[−], GFAP⁺ process-bearing cells. † Oligodendrocytes were defined as GC⁺, GFAP[−] process-bearing cells.

later, however, approximately equal numbers of type-1 astrocytes were found all along the nerve. This argues against the existence of a general chiasm-to-retina gradient of glial maturation in the optic nerve.

The gradual spread of O-2A progenitors could be due to migration of these cells along the developing optic nerve from the chiasmal end towards the eye. To determine directly whether progenitors are migratory, we used time-lapse microcinematography to study optic nerve cultures. These studies demonstrated that O-2A progenitors are highly motile, unlike their differentiated progeny or other non-O-2A lineage cells present in the cultures. In these experiments O-2A progenitor cells, grown in medium conditioned by type-1 astrocytes (so as to promote division of progenitors, refs 4 and 5), migrated with an average speed of $21.4 \pm 1.6 \mu\text{m h}^{-1}$ (s.e.m., $n = 66$), calculated over a 12-h period; maximal speeds over this same time period reached $100 \mu\text{m h}^{-1}$ (Fig. 1). Cells moved in the direction of either of their bipolar processes, often reversing their direction to migrate back along their original paths. Transient periods of non-motility were often associated with a striking shuttling of the nucleus between the two ends of the cell.

Following division, the new progenitor cells migrated away in opposite directions, each following the original orientation of the processes of the bipolar parent cell. Migration in opposite directions meant that families of cells derived from a single progenitor frequently covered areas of $\geq 4,400 \mu\text{m}^2$ over a period of 5 days. However, migration ceased when progenitors differentiated into recognizable multipolar oligodendrocytes. Similarly, oligodendrocytes present in the cultures at the beginning of filming did not migrate; their only movement was related to the elongation or elaboration of their processes. In medium containing 10% FCS, type-2 astrocytes also appeared to be non-motile, suggesting that the only cell of this lineage capable of migration is the O-2A progenitor cell itself.

If the motile properties displayed by the progenitor *in vitro* are similar to those *in vivo* and do not change with age, then a

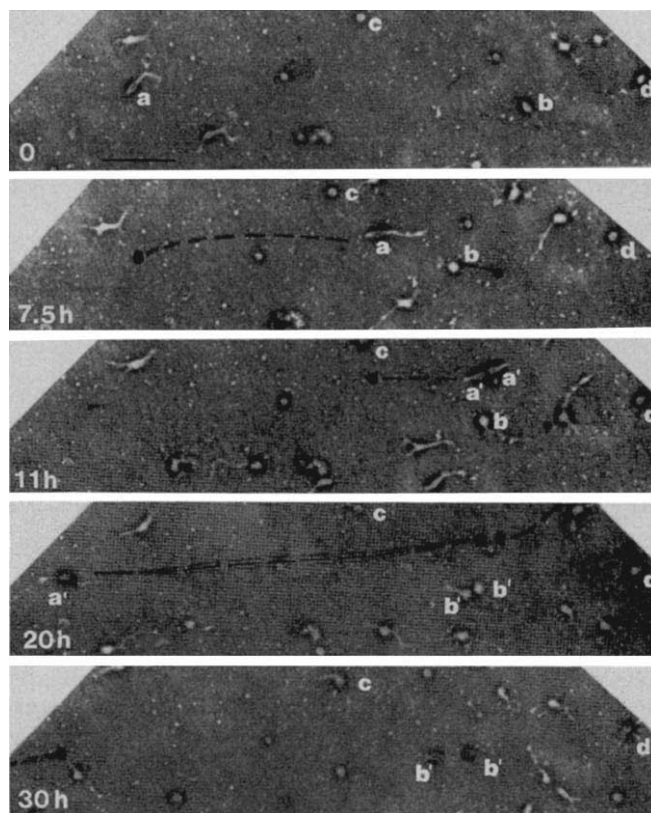


Fig. 1 O-2A progenitors are motile cells, but oligodendrocytes are not. Frames representing different times after an arbitrary 0 h were selected from 16-mm films of dissociated optic nerve cells to demonstrate the motility of bipolar progenitor cells (cells a and b) in contrast to the lack of motility of multipolar oligodendrocytes (cells c and d). Broken lines, track of migration; ●, position of cell in previous frame. In these experiments the O-2A progenitors showed a wide range of migration rates, as discussed in the text. In contrast, oligodendrocytes never showed migratory behaviour. Here, the progeny of dividing progenitor cell a (a') continue to be motile and migrate out of the frame whereas the progeny of dividing cell b (b') differentiate into non-motile oligodendrocytes. Bar, 100 µm.

Methods. Dissociated cells from P7 optic nerve were grown in chemically-defined medium conditioned by type-1 astrocytes. Purified type-1 astrocytes were plated in a ring around the periphery of 60-mm poly-L-lysine coated Petri dishes. After 24 h, 10,000 dissociated cells from the optic nerves of 7-day-old rats were plated in a 100-µl drop in the centre of the Petri dishes. Optic nerve cells were allowed to attach for 1 h, and then the cultures were fed with 1 ml of chemically-defined medium mixed with 1 ml of medium which had been conditioned by other purified astrocytes for 24 h (ref. 4). Cultures were maintained in a gassed incubator at 37 °C overnight before fields were chosen for filming. Fields of interest were chosen as those containing at least two identifiable bipolar O-2A progenitor cells. Cultures were filmed on Olympus microscopes adapted for time-lapse microcinematography and were maintained at 37 °C; one frame was photographed every 5 min. The O-2A progenitor cells in these cultures could be recognized by their characteristic bipolar morphology, in contrast to the multipolar process-bearing morphology of oligodendrocytes or the non-process-bearing morphology of type-1 astrocytes and meningeal cells. Parallel cultures growing on poly-L-lysine-coated coverslips were stained with cell-type-specific antibodies to confirm our morphological identification of cell-types. All process-bearing cells identified morphologically as oligodendrocytes were GC⁺, and all bipolar cells identified morphologically as O-2A progenitors were NSP-4⁺ and A2B5⁺. In addition, in all cases where oligodendrocytes were derived from dividing cells, the dividing cells initially expressed the characteristic bipolar morphology of the O-2A progenitor.

Table 3 Distribution of type-1 astrocytes in perinatal rat optic nerves

Age	Numbers of type-1 astrocytes per nerve segment			Total type-1 astrocytes per nerve
	Retinal	Middle	Chiasmal	
E16	278 ± 48	75 ± 19	43 ± 16	434
E17	855 ± 91	506 ± 42	198 ± 27	1,559
E19	2,072 ± 112	1,660 ± 108	1,873 ± 85	5,605
P0	4,139 ± 216	4,899 ± 240	3,894 ± 278	12,932

Cells were dissociated and double stained with anti-NSP-4 and anti-GFAP as in Table 1. Numbers (mean ± s.d.) were obtained by counting cells isolated from nerve segments and multiplying by the percentage of type-1 astrocytes (GFAP⁺ NSP-4⁻ cells without processes) as assessed by immunofluorescence in three or more experiments.

progenitor migrating at 20 $\mu\text{m h}^{-1}$ could traverse the 2.0 mm of the newborn optic nerve in about 4 days; in the E17 nerve, a progenitor could move from the middle segment into the retinal portion of the nerve, a distance of <0.4 mm, in less than a day.

There are several indications from past research that migration of oligodendrocyte-related cells occurs readily in organ culture and *in vivo*. First, cells migrating out of explants of both neonatal and adult optic nerves are capable of myelinating cerebellar explants⁶. In addition, when bits of normal neonatal mouse brain are transplanted into shiverer mice, which are unable to make myelin basic protein (MBP), MBP-containing myelin is found very long distances from the graft⁷. Moreover, a cephalocaudal infiltration of oligodendrocytes into irradiated regions of neonatal rat spinal cord leads to limited myelination of the rostral portion of the irradiated area^{8,9}. However, none of these studies provides information on the identity of the migratory cells responsible for myelination.

Our observations suggest that O-2A progenitor cells, rather than oligodendrocytes, are the cells capable of the long-distance migrations observed above⁶⁻⁹. More importantly, our *in vivo* studies suggest that such migrations may be the developmental mechanism by which areas destined for myelination, such as the optic nerve, become populated by the O-2A lineage. Progenitor cell migrations may occur widely during development of the CNS. A likely source of the optic nerve's progenitor cells is the base of the preoptic recess overlying the optic chiasm. This zone shows a concise period of proliferation beginning at E16, just after ganglion cell axons arrive at the chiasm and ending at E19 (J. Altman, personal communication). During this embryonic period the optic nerve, leading into the chiasm, becomes filled with glial cells whereas its extension beyond the chiasm, the optic tract, remains largely devoid of glia (data not shown). This suggests that progenitor cells from the optic chiasm migrate preferentially into the optic nerve rather than the tract.

If O-2A progenitor cells migrate from such germinal zones to where myelination is required, two critical questions arise: what is the substrate for migration and what prevents these cells from migrating into areas of the CNS that do not become myelinated, such as the retina in most mammalian species. Because progenitors appear in the optic nerve only after axons reach the chiasm¹⁰, these axons may guide migration of progenitors into the nerve. This could explain why lesions of newborn rat optic nerve drastically reduce the O-2A cell lineage in the nerve while having minimal effects on the type-1 astrocyte population¹¹. However, an additional factor is required to explain why myelination of the optic nerve ceases behind the eye in the retrobulbar area known as the lamina cribrosa¹²; the O-2A cell lineage is not present in the rat retina (unpublished data) although intraorbital portions of ganglion cell axons are known to be myelin competent¹³. Failure of the O-2A lineage to gain access to the retina points to the existence of a barrier early in development when progenitors first reach the lamina cribrosa; minor shifts in migratory rate, or delay or failure of barrier construction, might be important in those cases of con-

genital retinal myelination, such as occurs in the rabbit, where myelination continues uninterrupted from the optic nerve into the central region of the retina^{14,15}.

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1. Raff, M. C., Miller, R. H. & Noble, M. *Nature* **303**, 390-396 (1983).
2. Ffrench-Constant, C. & Raff, M. C. *Nature* **323**, 335-338 (1986).
3. Miller, R. H., David, S., Patel, R., Abney, E. R. & Raff, M. C. *Dev. Biol.* **111**, 35-41 (1985).
4. Noble, M. & Murray, K. *EMBO J.* **3**, 2243-2247 (1984).
5. Raff, M. C., Abney, E. R. & Fok-Seang, J. *Cell* **42**, 61-69 (1985).
6. Wolf, M. K., Brandenburg, M. C. & Billings-Gagliardi, S. J. *Neurosci.* **6**, 3731-3738 (1986).
7. Gumpel, M., Baumann, N., Raoul, M. & Jacque, C. *Neurosci. Lett.* **37**, 307-311 (1983).
8. Beal, J. A. & Hall, J. L. *J. Neuropath. Exp. Neurol.* **33**, 128-143 (1974).
9. Blakemore, W. F. & Patterson, R. C. *J. Neurocytol.* **4**, 573-585 (1975).
10. Lund, R. D. & Bunt, A. H. *J. comp. Neurol.* **165**, 247-264 (1976).
11. David, S., Miller, R. H., Patel, R. & Raff, M. C. *J. Neurocytol.* **13**, 961-974 (1984).
12. Hildebrand, C., Remahl, S. & Waxman, S. G. *J. Neurocytol.* **14**, 597-617 (1985).
13. Perry, H. V. & Hayes, L. *J. Neurocytol.* **14**, 297-307 (1985).
14. Marchesani, O. *Graefes Arch. Ophthalmol.* **117**, 575-605 (1926).
15. Berliner, M. L. *Arch. Ophthalmol.* **6**, 740-751 (1931).
16. Ranscht, B., Clapham, P. A., Price, J., Nobel, M. & Seifert, W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2709-2713 (1982).
17. Rougon, G., Hirsch, M. R., Hirn, M., Guenet, J. L. & Goridis, C. *Neuroscience* **10**, 511-520 (1983).
18. Bignami, A., Eng, L. F., Dahl, D. & Uyeda, C. T. *Brain Res.* **43**, 429-435 (1972).
19. Eisenbarth, G. S., Walsh, F. S. & Nirenberg, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4913-4917 (1979).
20. Bottenstein, J. E. & Sato, G. H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 514-517 (1979).

Correlation between the anaesthetic effect of halothane and saturable binding in brain

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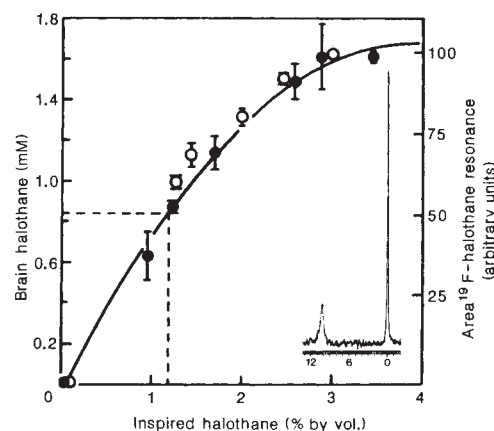
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Two theories of the molecular mechanism of volatile anaesthetic action suggest either that anaesthetics cause a generalized perturbation of neuronal membrane structure, probably through a non-specific interaction with membrane lipids^{1,2}, or that anaesthetics bind to sets of sites of appropriate molecular dimension on membrane proteins³⁻⁵. Based on the recent finding that fluorinated anaesthetics can be observed in animal tissue by ¹⁹F nuclear magnetic resonance (¹⁹F-NMR) spectroscopy⁶, we have used ¹⁹F-NMR to quantify the interaction between the volatile anaesthetic halothane and rat brain tissue. Steady-state brain halothane concentration was found to be a non-linear function of inspired concentration, with apparent saturation of brain occurring at inspired halothane concentrations above 2.5% by volume. Using a spin-echo pulse sequence it was found that halothane exists in two distinct chemical environments in brain, characterized by different spin-spin relaxation times (*T*₂), chemical shifts and kinetics of occupancy. Halothane concentration in one of these environments (*T*₂ = 3.6 ms) was saturated at ~2.5% inspired halothane; occupancy of this environment was found to correlate with the anaesthetic effect of the drug. In the other environment (*T*₂ = 43 ms), brain halothane concentration was a linear function of inspired concentration. These data suggest the existence of a saturable anaesthetic site for halothane in brain and do not support the concept that anaesthetics act by nonspecific membrane perturbation.

The ¹⁹F-NMR spectra of brain tissue from rats anaesthetized

Fig. 1 Brain halothane concentration as a function of inspired halothane concentration. •, Mean $\pm$ s.d. of six determinations of brain halothane concentration in excised tissue (three rats, left and right hemispheres); ○, mean ($\pm$ s.d.) steady state relative brain halothane concentration determined in three animals. Horizontal line, half-maximal brain concentration of halothane. This concentration was achieved at $\sim 1.2\%$ inspired halothane, corresponding to the anaesthetic ED_{50} of halothane in rats. Inset, a representative ^{19}F spectrum from these experiments. The feature at 0 p.p.m. corresponds to methoxyflurane in the external standard, and the resonance feature at 10.2 p.p.m. corresponds to halothane in brain.

Methods. Groups of three male Sprague-Dawley rats (250–300 g) were anaesthetized for 1 h with known inspired concentrations of halothane (as validated by gas chromatography)¹⁵ ranging from 0 to 4% by volume. The rats were decapitated and the left and right cerebral hemispheres were placed in separate gas-tight syringes. The brain tissue was injected into a sealed 5-mm glass capillary, and the capillaries were then inserted into a 10-mm NMR tube immersed in an external standard solution consisting of 1.42 mM methoxyflurane ($\text{CHCl}_2\text{—CF}_2\text{—OCH}_3$) in toluene d_8 -toluene: (1:2 by volume). Trace amounts of the paramagnetic relaxation agent chromium acetylacetonate were added to the external standard solution. Methoxyflurane in the external standard solution served both as a chemical shift reference and a concentration reference. NMR measurements were carried out at 10 °C using a Varian XL-300 multinuclear spectrometer (7.4T) equipped with a 10-mm probe. The ^{19}F resonances of halothane and methoxyflurane were observed at 282.2 MHz under steady-state equilibrium conditions (delays $>5T_1$ between successive 90° pulses) without proton decoupling. Quantitative analysis of brain halothane concentration was performed as follows: the ratio of the integrations of the brain halothane resonance to the methoxyflurane resonance was calculated to determine the number of fluorine atoms in the brain sample relative to the external standard. Account was then taken of the relative volumes (cross-sectional areas) of brain and external standard in the magnetic field, and for the number of fluorine atoms in halothane to determine the 'apparent molarity' of halothane in brain. To correct for inhomogeneity in the brain samples (bubbles, variable density of packing and so on) the apparent molarity was normalized for the absolute volume of brain tissue in the magnetic field. This was accomplished by observing the proton NMR signal of the samples and determining the ratio of the integrations of the water resonance in brain to the resonance of the methyl group of toluene in the external standard solution. As rat brain is known to be 84% water by volume¹⁶, the brain-water/methyl-group of toluene ratio was normalized to the water/methyl-group of toluene ratio obtained from a capillary containing 84% water. The apparent molarity of halothane in brain was corrected for this calculated volume factor, and the value obtained was considered brain halothane concentration. Adequate signal-to-noise ratio was achieved with 64 transients for ^{19}F spectra and 8 transients for ^1H spectra. For *in vivo* experiments, rats were anaesthetized with 1.25% halothane, allowed to ventilate spontaneously through a tracheostomy using a non-rebreathing system and positioned in the core of a Bruker CXP-200 NMR spectrometer. Five concentrations of halothane (1.25, 1.5, 2.0, 2.5 and 3.0%) were administered sequentially, each for 30 min, using a calibrated temperature-compensated vaporizer. The ^{19}F spectra were acquired from a surface coil antenna centred over the calvaria. A 4-mm microsphere containing 0.5% hexafluorobenzene (in d_6 -benzene, 65 mM in chromium acetylacetonate) was placed in the centre of the coil and served as a chemical shift and integration reference. The following acquisition parameters were used: 30,000 Hz sweep width, 0.2 s acquisition time, 15–20 μs pulse width (selected to give a 180° pulse to the hexafluorobenzene standard) and 600 transients; proton decoupling was not used. The area of the halothane resonance was seen to reach a steady-state following ~ 20 min at any given concentration. Accordingly, steady-state levels were determined from the spectra collected between 20 and 30 min exposure to each concentration. The integral values obtained at 3% inspired halothane were arbitrarily given the value of 100 and integral values obtained at lower concentrations are expressed relative to 100.



with halothane ($\text{CF}_3\text{—CHBrCl}$) show a single resonance for the trifluoromethyl group of halothane at 10.2 p.p.m. (relative to the resonance of the difluoromethylene group of methoxyflurane ($\text{CHCl}_2\text{—CF}_2\text{—OCH}_3$) in the external standard, arbitrarily defined as 0 p.p.m.) (Fig. 1). The halothane resonance is similar in terms of chemical shift and line width (80–120 Hz) to the halothane resonance observed in rabbit brain by Wyrwicz⁷. To quantify the relationship between inspired and brain halothane concentrations, halothane concentration was measured in the excised cerebral hemispheres of rats anaesthetized at various inspired halothane concentrations for 1 h. Brain halothane concentration was a linear function of inspired concentration from 0–2.5% halothane (Fig. 1). At higher inspired concentrations rat brain seemed to become saturated with halothane. The results of the excised-tissue studies were confirmed by *in vivo* ^{19}F -NMR spectroscopic measurement of relative brain halothane concentration as a function of inspired concentration. In these experiments brain again seemed to become saturated with halothane at inspired concentrations above 2.5% (v/v) (Fig. 1).

As high inspired concentrations of halothane are associated with respiratory and cardiac depression^{8,9}, which might impair anaesthetic uptake, we also measured plasma halothane concentration. Arterial blood was withdrawn anaerobically from animals anaesthetized with halothane for 1 h, and plasma was prepared by centrifugation. Plasma halothane concentration was then measured by ^{19}F -NMR spectroscopy, using methoxyflurane as an external concentration reference. The concentration of halothane in plasma was found to increase continuously over the same regime of inspired concentration in which brain tissue saturated. Notably, plasma halothane concentrations were sub-

stantially lower than brain concentrations; at 1.7%, 2.7% and 3.6% inspired concentration, plasma halothane values are 0.20 ± 0.01 mM, 0.25 ± 0.03 mM and 0.33 ± 0.02 mM (mean of three determinations $\pm$ s.d.), respectively. This finding rules out the possibility that saturation of brain with halothane is the consequence of impaired anaesthetic uptake or plasma saturation at high inspired concentrations.

NMR spectroscopy can also be used to provide information about the chemical environment in which a molecule resides. Specifically, the spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) provide information about the rotational freedom (correlation time) of a molecule. To gain further information about the interaction of halothane with brain tissue, we measured T_1 and T_2 of the halothane ^{19}F resonance in brain. T_1 was measured by the inversion recovery method¹⁰. Halothane in brain had a T_1 value of 0.45 s as compared to a T_1 value of 4.0 s for halothane (10 mM) in chloroform. The Carr-Purcell-Meiboom-Gill sequence (spin-echo method) was used to measure T_2 (ref. 11). Measurements of T_2 revealed two distinct T_2 values for halothane in brain, one of 3.6 ± 0.6 ms (mean of five determinations $\pm$ s.d.), and one of 43 ± 8 ms (Fig. 2). For comparison, the T_2 of halothane (10 mM) in solution is 1.5 s. These data indicate that halothane is highly immobilized by its interaction with brain tissue, and that the halothane molecule exists in two distinct chemical microenvironments in brain. In one of the environments ($T_2 = 3.6$ ms), the halothane molecule is more highly immobilized than in the other environment ($T_2 = 43$ ms). These two environments are also characterized by distinct spectral differences. As the evolution time in the T_2 (spin-echo) sequence was increased, the halothane resonance pro-

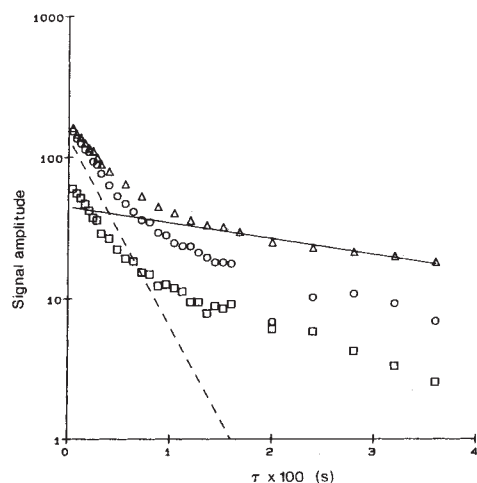


Fig. 2 Representative spin-echo curves of the ^{19}F -halothane resonance in rat brain, as determined by the Carr-Purcell-Meiboom-Gill method. Animals were anaesthetized for 1 h at inspired halothane concentrations of 3.95% (Δ), 2.87% ($\circ$) and 0.95% ($\square$), and immediately decapitated. The cerebral hemispheres from each rat were placed in a syringe and injected through polyethylene tubing (PE-260) into a 10 mm NMR tube containing 1.2 ml of 0.15% NaCl in D_2O . The D_2O served both as a locking signal and as a suspension medium for the brain tissue. The ^{19}F spectra were obtained at 10°C , using a $90^\circ\text{-}\tau\text{-(}180^\circ\text{-}2\tau\text{)}_n$ pulse sequence. Approximately 25 echo evolution times ranging from 0.0004–0.036 s, were used in each experiment and 200 transients were collected at each evolution time. The graph shows the natural logarithm of the intensity of the ^{19}F -halothane signal as a function of echo evolution time (referred to here as τ). Note that the intensity decreases with delay along a time course than can be well described by the sum of two exponential components. The more rapidly declining component shows little or no increase between 2.87% and 3.95% inspired halothane, whereas the slowly declining component increases steadily as the inspired halothane concentration increases (Fig. 3). The T_2 (spin-spin relaxation time) values were determined from these plots by a curve stripping method. The longer T_2 component ($T_2 = 43$ ms) was determined by a linear regression of semilogarithmically transformed data collected at τ values greater than 15 ms. Subtraction of this component from the raw echo amplitude data yielded the shorter T_2 curve ($T_2 = 3.6$ ms). Finally, T_2 values were determined as the reciprocal of the negative slope of the two lines derived from curve stripping.

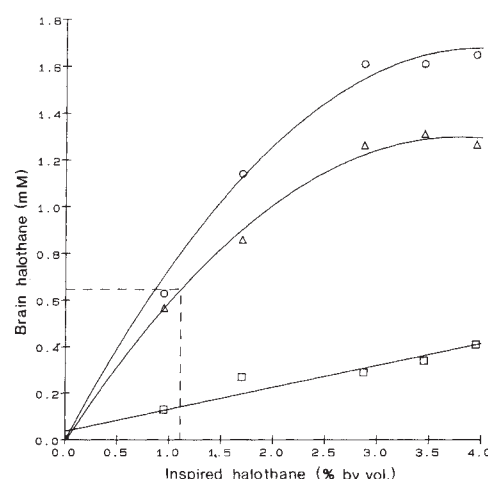


Fig. 3 Halothane concentrations in the two microenvironments of brain (as defined by T_2) as a function of inspired concentration. Total brain halothane concentration ($\circ$) was determined at various inspired concentrations as in Fig. 1. To determine halothane concentration in each of the two microenvironments, spin-echo decay curves were generated from the brain of rats anaesthetized (1 h) at various inspired concentrations. The concentration of halothane in the environment with $T_2 = 43$ ms ($\square$) was determined from the y-intercept values for two exponential components fitted to plots such as those shown in Fig. 2. The concentration of halothane in the environment with $T_2 = 3.6$ ms (Δ) was determined by subtraction of the y-intercept value of the longer T_2 component from the total y-intercept value. These data illustrate that the environment in which halothane is more highly immobilized ($T_2 = 3.6$ ms) becomes saturated at inspired concentrations $>2.5\%$. The other environment ($T_2 = 43$ ms) is populated in a linear fashion up to the highest inspired halothane concentration studied (4%). As illustrated by the horizontal line, half-maximal concentration of halothane in the saturable environment is achieved at an inspired concentration ($\approx 1.2\%$) equal to the anaesthetic ED_{50} of halothane in rats.

gressively narrowed from 80–120 Hz to ~ 20 Hz. The narrow feature (20 Hz) was more highly shielded from the magnetic field than the broad feature, with a chemical shift difference between the two features of ~ 0.1 – 0.2 p.p.m.

To characterize further the two environments in brain, T_2 measurements were performed at variable temperature (5°C , 15°C and 25°C). In the environment in which halothane is highly immobilized, T_2 increases with increasing temperature ($T_2 = 2.7$ ms at 5°C , $T_2 = 4.8$ ms at 25°C), behaviour consistent with dipolar-dependent relaxation. In the other environment T_2 paradoxically decreases with increasing temperature ($T_2 = 40$ ms at 5°C , $T_2 = 14$ ms at 25°C). This behaviour suggests that the measured long T_2 represents an exchange process of halothane between a site in brain and the aqueous phase¹². It is unlikely that either of the two observable T_2 values represents halothane purely in an aqueous phase, which would give rise to a very narrow line (<6 Hz) and a T_2 value of ~ 1.5 s. It is also unlikely that either of these two T_2 values represents halothane in blood, both because blood represents a small proportion of brain volume and because the halothane resonance in whole blood is extremely broad (>250 Hz). The environment with $T_2 = 43$ ms may, however, represent an interaction of halothane with membrane lipid. Addition of halothane to highly concentrated liposomes prepared from rat brain lipids reveals a resonance of line width 20 Hz with a single T_2 value of 30–60 ms. This is consistent

with a previous report¹³ of a similar spectral feature observed when phosphatidylcholine vesicles were treated with halothane. The T_2 of halothane in liposomes varies inversely with liposome concentration, suggesting that the measured T_2 represents an exchange process between liposomes and the aqueous phase. Consistent with an exchange process, the T_2 of halothane in liposomes paradoxically decreases with increasing temperature. The similar behaviour of halothane in liposomes and halothane in the long T_2 (43 ms) site in brain suggests that the long T_2 site in brain may represent an interaction of halothane with membrane lipid. These data clearly indicate that the short T_2 site in brain cannot be accounted for by a nonspecific lipid interaction. It is unclear from the present data whether the two halothane environments represent different chemical milieux in plasma membranes of the same cell type, or whether the environments represent localization in distinct anatomical regions (for example, white and grey matter) or cell types.

Having defined two environments for halothane in brain, we measured the concentration of halothane in these two pools as a function of inspired anaesthetic concentration (1–4%). The environment in which halothane is most highly immobilized ($T_2 = 3.6$ ms) is saturated at an inspired halothane concentration of 2.0–2.5%, whereas halothane concentration in the other environment ($T_2 = 43$ ms) is a linear function of inspired concentration. Half-maximal occupancy of the saturable environment was found to occur at the same inspired concentration (1.2% by volume) as that required to prevent response to noxious stimuli in 50% of rats¹⁴ (Fig. 3), suggesting that occupancy of the saturable environment might be responsible for the anaesthetic effect of halothane. To examine this possibility further, we studied rats during the induction of anaesthesia. Rats

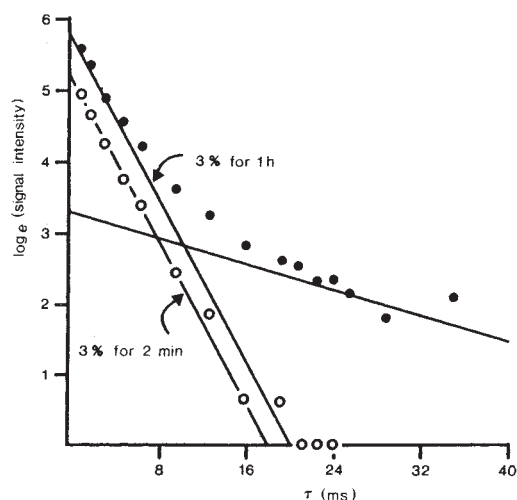


Fig. 4 Representative spin-echo decay curves of the ^{19}F -halothane resonance in rat brain from rats exposed to 3% halothane for 2 min (O) or 1 h (●). Experiments were performed and analysed as in Fig. 2. The results illustrate that in rats anaesthetized with 3% halothane for 2 min, halothane exists almost exclusively in the environment with $T_2 = 3.6$ ms. Following a 1-h exposure to 3% halothane, the anaesthetic molecules occupy two distinct environments. This experiment was performed five times with similar results. Note that the y axis is natural logarithm of signal intensity.

exposed to 3% halothane lose their response to noxious stimuli after 120–130 s (ref. 14). Accordingly, rats were anaesthetized for 120 s with 3% halothane and promptly decapitated. Brain halothane concentration in these rats (0.64 ± 0.04 mM) was ~50% of the maximal concentration in the saturable environment (~1.2 mM). Measurement of T_2 in brains from these rats showed that >95% of the anaesthetic was in the environment with $T_2 = 3.6$ ms, with barely detectable occupancy of the non-saturable site (Fig. 4). These results indicate that ~50% occupancy of the saturable site is sufficient to produce the anaesthetic effect, and that uptake of halothane into the two environments described occurs at different rates.

These results indicate that halothane exists in two chemical environments in rat brain. At sublethal inspired halothane concentrations, the majority of the anaesthetic is found in a saturable environment in which the anaesthetic molecules have greatly reduced rotational freedom. Occupancy of this environment is associated with the anaesthetic effect of the drug. This environment is unlikely to represent a single homogenous protein receptor, because brain becomes saturated at such high concentrations of halothane. This saturable site is more likely to represent an interaction between the drug and either a degenerate site or sites common to many membrane proteins or a specific membrane lipid. Halothane also exists in a second environment in brain that is consistent with a nonspecific lipid partition, and is apparently unrelated to the anaesthetic effect. Chemical characterization of the saturable site for halothane should provide much insight into the molecular mechanism of anaesthetic action.

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1. Miller, K. W. & Pang, K. Y. *Nature* **263**, 253–255 (1976).
2. Miller, K. W. *Int. Rev. Neurobiol.* **27**, 1–61 (1985).
3. Richards, C. D. *et al. Nature* **276**, 775–779 (1978).

4. Franks, N. P. & Lieb, W. R. *Nature* **300**, 487–493 (1982).
5. Halsey, M. J., Wardley-Smith, B. & Green, C. J. *Br. J. Anaesth.* **50**, 1091–1097 (1978).
6. Wyrwicz, A. M., Li, Y., Schofield, J. C. & Burt, C. T. *FEBS Lett.* **162**, 334–338 (1983).
7. Wyrwicz, A. M., Pszeny, M. H., Tillman, P. C., Gordon, R. E. & Martin, P. A. *Science* **222**, 428–430 (1983).
8. Deutsch, S. *et al. Anesthesiology* **23**, 631–638 (1962).
9. Eger, E. I. III *et al. Anesthesiology* **32**, 396–409 (1970).
10. Vold, R. L., Waugh, J. S., Klein, M. P. & Phelps, D. E. *J. chem. Phys.* **48**, 3831–3832 (1968).
11. Meiboom, S. & Gill, D. *Rev. Sci. Instrum.* **29**, 688–691 (1958).
12. Resing, H. A. & Garroway, A. N. in *Magnetic Resonance in Colloid and Interface Science* (eds Resing, H. A. & Wade, C. G.) 516–529 (Am. Chem. Soc., Washington, 1976).
13. Koehler, L. S., Fossel, E. T. & Koehler, K. A. *Biochemistry* **16**, 3700–3707 (1977).
14. Evers, A. S., Elliot, W. J., Lefkowitz, J. B. & Needleman, P. *J. clin. Invest.* **77**, 1028–1038 (1986).
15. Heaven, J. E., Friedhoff, J. & Haschke, R. *Anesthesiology* **45**, 654–655 (1976).
16. Lowry, O. H., Hastings, A. B., McCay, C. M. & Brown, A. N. *J. Geront.* **1**, 345–357 (1946).

Correlation between the ATPase and microtubule translocating activities of sea urchin egg kinesin

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Coupling between ATP hydrolysis and microtubule movement was demonstrated several years ago in flagellar axonemes^{1,2} and subsequent studies suggest that the relevant microtubule motor, dynein, uses ATP to drive microtubule sliding by a cross-bridge mechanism analogous to that of myosin in muscles^{3,4}. Kinesin⁵, a microtubule-based motility protein which may participate in organelle transport and mitosis⁶, binds microtubules in a nucleotide-sensitive manner^{5,7,8}, and requires hydrolysable nucleotides to translocate microtubules over a glass surface^{9,10}. Recently, neuronal kinesin was shown to possess microtubule-activated ATPase activity^{11,12} although coupling between ATP hydrolysis and motility was not demonstrated. Here we report that sea urchin egg kinesin, prepared either with or without a 5'-adenylyl imidodiphosphate (AMPPNP)-induced microtubule binding step, also possesses significant microtubule-activated ATPase activity when Mg-ATP is used as a substrate. This ATPase activity is inhibited in a dose-dependent manner by addition of Mg-free ATP, by chelation of Mg^{2+} with EDTA, by addition of Na_3VO_4 , or by addition of AMPPNP with or without Mg^{2+} . Addition of these same reagents also inhibits the microtubule-translocating activities of sea urchin egg kinesin in a dose-dependent manner, supporting the hypothesis that kinesin-driven motility is coupled to the microtubule-activated Mg^{2+} -ATPase activity.

Sea urchin egg kinesin displays microtubule-activated ATPase activity under appropriate assay conditions (Table 1). At room temperature (~25 °C), the Mg^{2+} -ATPase activities of egg kinesin were low or absent when assayed in the absence of microtubules (Table 1; mean activity $2.2 \text{ nmol min}^{-1} \text{ mg}^{-1}$) as observed by Vale *et al.*⁵ for neuronal kinesin. Increasing the assay temperature increased the basal egg kinesin ATPase activity (for example, from background levels at room temperature to $60.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ at 37 °C in one experiment). We consistently observed that the low Mg^{2+} -ATPase activity of egg kinesin was activated significantly by taxol-stabilized bovine brain microtubules, from background levels to between 20 and $60 \text{ nmol min}^{-1} \text{ mg}^{-1}$ at room temperature (Table 1; mean = $39.0 \text{ nmol min}^{-1} \text{ mg}^{-1}$, an ~18-fold activation). Increasing the temperature of assay to 37 °C also increased the microtubule-activated Mg^{2+} -ATPase activity (from $32.9 \text{ nmol min}^{-1} \text{ mg}^{-1}$ at

Table 1 Microtubule-activated Mg^{2+} -ATPase activity of sea urchin egg kinesin

Method 1 (AMPPNP)	Preparation 1		Preparation 2		Preparation 3	
	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹
Kinesin	0.64	7.90	-0.063	-0.58	-0.14	-2.50
Microtubules	0.65	—	0.19	—	0.69	—
Kinesin + microtubules	5.28 - 0.65 = 4.63	57.50	2.19 - 0.19 = 2.0	23.50	2.5 - 0.69 = 1.81	32.90
Method 2 (apyrase)	Preparation 1		Preparation 2		Preparation 3	
	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹	nmol min ⁻¹ ml ⁻¹	nmol min ⁻¹ mg ⁻¹
Kinesin	0.28	1.75	0.44	8.70	-0.14	-2.20
Microtubules	0.63	—	0.36	—	-0.17	—
Kinesin + microtubules	3.9 - 0.63 = 3.30	20.60	3.2 - 0.36 = 2.84	56.30	2.53 + 0.17 = 2.70	42.90

Materials, general biochemical procedures, preparation of unfertilized sea urchin egg taxol-assembled microtubules and bovine brain tubulin²³, were as described previously^{8,10,19}. Kinesin was isolated from sea urchin egg extract supernatants prepared by homogenization of packed, dejellied eggs in 2 vol PMEG extraction buffer (0.9 M glycerol, 0.1 M PIPES pH 6.96, 5 mM EGTA, 2.5 mM MgSO_4 , 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg ml⁻¹ soybean trypsin inhibitor, 10 $\mu\text{g ml}^{-1}$ leupeptin, aprotinin, and pepstatin) and centrifugation at 55,000g for 30 min, then at 150,000g for 60 min, using a Beckman Ti50.2 rotor (all steps at 0–4 °C). The extract was subsequently incubated with hexokinase (10 units ml⁻¹) and 50 mM glucose²⁴ to deplete ATP, and centrifuged at 100,000g to pellet actomyosin, leaving kinesin and microtubule protein in solution. Microtubule assembly in this supernatant was induced by the addition of 1 mM GTP plus 20 μM taxol and incubation at room temperature for 15 min^{25,19}. The extract was then supplemented with 1 mM AMPPNP plus 0.1 mM ATP (method 1) or 5 units ml⁻¹ apyrase (method 2), to enhance microtubule binding by kinesin, incubated 20 min and centrifuged over a 15% sucrose cushion in a Sorvall HB4 swinging bucket rotor at 23,000g, 60 min, 10 °C to pellet the kinesin-microtubule complexes. The pellets were resuspended and pelleted (45,000g, 20 min) in extraction buffer containing 0.1 mM GTP, resuspended in 0.1 M KCl plus 10 mM Mg-ATP (ATP extract) and held overnight at 0 °C. A further 5 mM Mg-ATP was added before centrifugation at 45,000g for 20 min, in a Beckman Ti50 rotor. The extracted MAP supernatants were fractionated by Biogel A5M chromatography in PMEG containing 1 mM ATP (ref. 8). Fractions were tested for microtubule-translocating activity by video microscopy as in Fig. 2, and analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and anti-kinesin Western blotting^{8,10}. The peak of kinesin activity constituted the fractions that would support microtubule movement at $\geq 0.4 \mu\text{m s}^{-1}$, which were pooled for further analysis. From 10 ml packed sea urchin eggs we obtained ~375 μg kinesin by method 1 and 450 μg kinesin by method 2. ATPase assays were performed at room temperature (~25 °C) by modifications of the method of Seals *et al.*²⁶. Biogel-A5M-fractionated egg kinesin in PMEG extraction buffer (200–300 μl) was mixed with 40 μl bovine brain microtubules at 10 mg ml⁻¹ (phosphocellulose-chromatographed tubulin²³, incubated in 20 μM taxol, 0.5 mM GTP for ≥ 2 h at room temperature) or with 40 μl buffer, plus 1 μl of 10 mM taxol. PMEG extraction buffer was added to each assay tube, to give a final volume 380 μl , and the mixture was incubated for ~10 min at room temperature. To start the reaction, 20 μl of 50 mM [γ -³²P] Mg-ATP with specific activity 10,000–40,000 c.p.m. nmol ATP, were added. Aliquots (100 μl) were removed at a minimum of three of the following time points (depending on the particular assay) 0, 5, 10, 15, 20, 30 or 60 min, and the reaction was quenched in 30 μl 10% SDS. Liberated phosphate was reacted with 75 μl phosphate reagent (4 vols 5N H_2SO_4 /5% ammonium molybdate: 1 vol. 0.1 M silicotungstic acid) and the resulting ³²P-labelled phosphomolybdate complex was extracted with 1 ml of a 65% : 35% (v/v) xylene isobutanol mixture. After centrifugation in a microfuge to separate organic and aqueous phases, 0.7 ml organic phase containing the ³²P phosphomolybdate was analysed by scintillation counting. Each preparation was assayed several times and typical results are shown. Note the variability in specific activities. We assume that the low negative values reflect experimental error.

room temperature to 113.5 nmol min⁻¹ mg⁻¹ at 37 °C in one experiment). The rate of microtubule-activated Mg-ATP hydrolysis by egg kinesin is lower than the values reported for neuronal preparations^{11,12}, but is comparable to the actin-activated Mg^{2+} -ATPase activity of phosphorylated vertebrate smooth muscle myosin (46–51 nmol min⁻¹ mg⁻¹ at 24 °C; ref. 13), which moves beads along actin filament bundles at 0.15–0.4 $\mu\text{m sec}^{-1}$ (ref. 14). This is consistent with the hypothesis that the microtubule-activated ATPase activity of egg kinesin can support translocation of beads along microtubules or microtubules over glass at 0.5 $\mu\text{m s}^{-1}$.

Enzymatically active egg kinesin was obtained using two different procedures (Table 1), both involving sedimentation of kinesin-microtubule complexes from egg high-speed extract supernatants, followed by Mg-ATP-dependent extraction of kinesin from the complex and Biogel A5M chromatography. Method 1 used AMPPNP to promote formation of the kinesin-microtubule complex whereas method 2 used apyrase-catalysed ATP-depletion (tripolyphosphate¹¹ being relatively ineffective in this system). Using either method, both microtubule-translocating activity and microtubule-activated ATPase activity co-eluted from Biogel A5M columns with the egg kinesin polypeptide of relative molecular mass 130,000 (M_r 130K) plus a number of other, minor polypeptides (see for example Fig. 3d, lane 2 of ref. 8). No significant difference in the motility or ATPase activities was found using either of these two procedures (to be described in detail elsewhere).

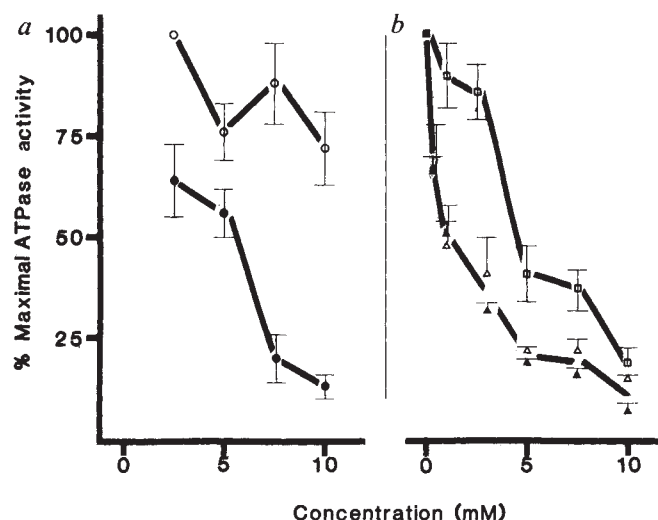
The conditions for assaying ATPase activity (Table 1) were based on previous characterization of the microtubule-trans-

locating properties of egg kinesin¹⁰. PMEG extraction buffer (see Table 1) works well in motility assays and was used throughout. Because the sea urchins used for these studies (*Strongylocentrotus purpuratus*) are poikilotherms which do not survive well at temperatures above 23.5 °C (ref. 15), the ATPase assays were routinely performed at room temperature. The microtubule-translocating activity of egg kinesin requires both Mg^{2+} and ATP; concentrations of nucleotide sufficient to chelate Mg^{2+} appeared to reduce motility¹⁰. Therefore, for the studies described here (Table 1), we routinely added equimolar MgSO_4 with the ATP substrate.

The microtubule-activated ATPase activity of egg kinesin is inhibited by adding Mg-free ATP (Fig. 1a). With addition of increasing concentrations of Mg-ATP, the microtubule-activated ATPase activity remained high (between 70% and 100% maximal activity), but a large decrease in activity was observed using Mg-free ATP; following addition of Mg-free ATP, to produce final total concentrations of 10 mM ATP plus 2.5 mM Mg in the assay, the microtubule-activated ATPase activities were <15% of control values (Fig. 1a). Similarly, when increasing concentrations of EDTA were added to the assay to chelate Mg^{2+} , a concomitant decrease in ATPase activity was observed (Fig. 1b). Although we do not know the exact concentrations of the different ionic species of ATP present in the final assay buffer (Fig. 1), these results suggest that Mg-ATP is an egg kinesin substrate and that Mg-free ATP acts as an inhibitor of the microtubule-activated Mg^{2+} -ATPase. Interestingly, Mg-free ATP has been reported to act as a competitive inhibitor of the dynein ATPase, where Mg-ATP is an effective substrate¹⁶. Low

Fig. 1. Inhibition of microtubule-activated ATPase activity of sea urchin egg kinesin. Bars, standard deviations. *a*, ATPase activities following addition of Mg-ATP (○) and Mg-free ATP (●). *b* Shows the results of adding the inhibitors EDTA (□), Mg-AMPPNP (△), and Mg-free AMPPNP (▲), with 2.5 mM Mg-ATP added as substrate.

Methods. ATPase activities were determined in PMEG extraction buffer (contains 2.5 mM Mg^{2+}) at room temperature in the presence of 1 mg ml⁻¹ taxol-stabilized bovine brain microtubules. Appropriate volumes of stock solutions or equal volumes of buffer were added to give the desired concentrations. Abscissa concentrations in *a* refer to total concentration of ATP; we have not calculated the final concentrations of individual ionic species $Mg\text{-ATP}^{2-}$, $HATP^{3-}$, ATP^{4-} and so on in the assay solution. Stocks of Mg-free [$\gamma\text{-}^{32}P$]ATP (50 mM) were made from 0.1 M ATP (disodium salt, Sigma, adjusted to pH 7.0 with NaOH) 3 μ M [$\gamma\text{-}^{32}P$]ATP (tetraethylammonium salt at 10 mCi ml⁻¹, Dupont) distilled water (10 vols:2 vols:8 vols). Stocks of [$\gamma\text{-}^{32}P$]Mg-ATP (50 mM) contained 1 vol 1M $MgSO_4$ in place of 1 vol distilled water. Abscissa concentrations in *b* refer to total added inhibitor concentrations, and all samples in (*b*) contained 2.5 mM added [$\gamma\text{-}^{32}P$]Mg-ATP as well. Stocks of 0.1 M AMPPNP (tetralithium salt, Sigma) and 0.2 M EDTA (disodium dihydrate; Sigma) were adjusted to pH 7.0 with NaOH. Na_3VO_4 (Aldrich) stocks were made exactly as described by Goodno²⁷. The results represent pooled data from several preparations. For clarity, in some cases the error bars are drawn in one direction only.



concentrations of AMPPNP inhibited the ATPase activity, whether or not it was added with equimolar concentrations of Mg^{2+} (Fig. 1*b*). Na_3VO_4 also inhibits the microtubule-activated Mg^{2+} -ATPase activity of egg kinesin in a dose-dependent manner (not shown), although egg kinesin is much less sensitive than dynein to the effects of vanadate (420 μ M Na_3VO_4 causes a 50% reduction in the microtubule-activated Mg^{2+} -ATPase activity of egg kinesin).

Previously it was observed that egg kinesin displayed low ATPase activities when assayed in the presence or absence of microtubules^{8,10}. In those studies^{8,10} high concentrations (up to 10 mM) of Mg-free ATP were usually added to the assay buffer (which contains 2.5 mM $MgSO_4$) to minimize the effects of any contaminating AMPPNP. The high concentrations of Mg-free ATP probably contributed to the low specific activities observed, although we cannot rule out the influence of other factors. The variable rates of ATP hydrolysis reported by different workers for neuronal kinesin^{5,11,12,17,18} may depend upon a number of factors, such as the method of protein isolation, the presence or absence of inhibitory or activating cofactors, or the conditions of assay.

To quantitate the effects of the inhibitors on microtubule translocation, microtubule velocities in the presence of various inhibitor concentrations were measured in parallel with the ATPase assays. Measurements were made in real time on live images from a video-enhanced microscope^{5,9,10} connected to a computer for calculation of velocities (Fig. 2). The velocity of microtubule gliding remained approximately constant when equimolar $MgSO_4$ was added with the ATP substrate. However, the velocity of kinesin-dependent microtubule translocation was inhibited in a dose-dependent manner by addition of Mg-free ATP, or by adding EDTA to chelate Mg^{2+} (Fig. 2). These data agree with the results of the ATPase assay and suggest that a divalent metal ion cofactor is required for the native ATP substrate, and that Mg-free ATP inhibits the activity of egg kinesin. AMPPNP (added with or without equimolar $MgSO_4$; Fig. 2*b*) and Na_3VO_4 (data not shown) also inhibited microtubule translocation in a dose-dependent manner. Of the five inhibitors studied, Na_3VO_4 was the most potent, causing a 50% reduction in translocation velocity at ~ 60 μ M, whereas similar inhibition by AMPPNP, EDTA and Mg-free ATP were seen on addition of approximately 450 μ M, 1.7 mM, and 3 mM respectively.

There is a direct correlation between ATPase activity and

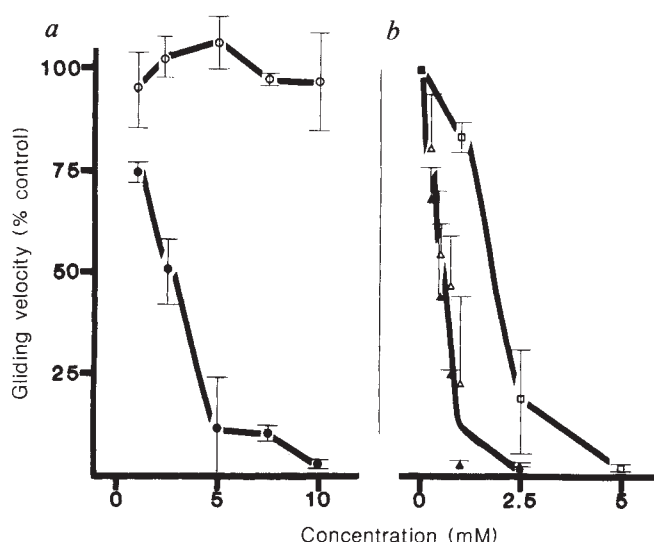
microtubule translocation with respect to the apparent relative efficacy of the inhibitors ($Na_3VO_4 > AMPPNP > EDTA > Mg\text{-free ATP}$). However, the concentration of inhibitor required to inhibit motility by 50% was lower than the concentration required to produce a similar effect on the ATPase activity; the microtubule-activated ATPase activity was reduced by 50% with addition of ~ 420 μ M Na_3VO_4 , 1 mM AMPPNP, 4.5 mM EDTA and 5.4 mM Mg-free ATP.

The differences in the inhibitor concentrations required to inhibit motility and ATPase to the same extent probably reflect different properties of the ATPase and motility assays. For example, microtubule translocation over a glass surface requires a minimum critical concentration of active, favourably oriented kinesin molecules adsorbed to the coverslip, which in turn depends upon factors such as the concentration of kinesin in solution during adsorption and the duration of incubation. We observe that a critical concentration of 20–40 μ g ml⁻¹ sea urchin egg kinesin (during adsorption) is required for motility. Moreover, the efficacy of the inhibitors in the motility assay is dependent upon the kinesin concentration above this critical concentration. For example, when a 150 μ g ml⁻¹ kinesin solution was adsorbed onto the coverslip, 660 μ M AMPPNP caused a 50% reduction in microtubule gliding velocity, whereas only 360 μ M AMPPNP was required for 50% inhibition of motility when 75 μ g ml⁻¹ kinesin was used. Microtubule-activated Mg-ATP hydrolysis, in contrast, probably occurs in solution and is relatively insensitive to the concentration of kinesin. When kinesin was diluted twofold, a 50% inhibition of the ATPase activity still required 0.9 times the AMPPNP concentration that caused 50% inhibition with undiluted kinesin. Furthermore, analysis of the microtubule binding characteristics of kinesin show that EDTA, Na_3VO_4 and AMPPNP induce the formation of a stable rigor-like kinesin-microtubule complex (refs 7, 8, 17 and unpublished observations). Such inhibitors could mediate strong binding of microtubules to glass surfaces by kinesin 'crossbridges', an effect which may potentiate the inhibition of microtubule gliding.

Although we have not directly demonstrated that the ATPase activity of sea urchin egg kinesin depends upon the exertion of mechanical force, our results strongly suggest that microtubule translocation and ATP hydrolysis are coupled because first, the ATPase activity is markedly stimulated by microtubules; second, microtubule translocation and the microtubule-activated

Fig. 2 Inhibition of kinesin-dependent microtubule translocation. The velocities of microtubule-gliding were measured using a video-enhanced microscope system with computer-assisted analysis as described below. A minimum of 10–15 microtubules were measured at each concentration for each kinesin preparation and results shown represent the average measurements combined from 2–3 separate kinesin preparations. Velocities are displayed as a percentage of the mean velocity of a control set of microtubules, with 2.5 mM Mg-ATP added, measured for each set of treatments. Mean velocity for the control microtubules was $0.57 \pm 0.05 \mu\text{m s}^{-1}$. *a*, Results using varying concentrations of added Mg-ATP (○) or Mg-free ATP (●). *b*, Results using varying concentrations of added EDTA (□), Mg-AMPPNP (△), and Mg-free AMPPNP (▲). Preparations in *a* contained the total ATP concentrations stated on the abscissa, whereas all preparations in *b* contained 2.5 mM added Mg-ATP plus the indicated amount of inhibitor. No significant difference was observed between AMPPNP and Mg-AMPPNP, and the line drawn is the average between the two sets of AMPPNP measurements.

Methods. The 3–5 peak fractions of Biogel A5M fractionated kinesin were pooled and used in these motility assays, and the assays performed in parallel with the ATPase assays. Pooled kinesin (15 μl) was spread onto a No. 1 coverslip and allowed to adsorb for at least 20 min in a humidified chamber. After adsorption, 2 μl of $\sim 0.2 \text{ mg ml}^{-1}$ microtubules (using bovine brain tubulin purified by phosphocellulose chromatography²³ and polymerized in 1 mM GTP and 20 μM taxol) were added, along with 0.5–2.0 μl each of nucleotide and/or inhibitor solution (made up as stock solutions of 1 mM–100 mM) to obtain a final volume of $\sim 20 \mu\text{l}$. Concentrations indicated in the graphs are final added concentrations (see Fig. 1 legend). The coverslip was inverted and sealed to a microscope slide with VALAP (vaseline/lanolin/paraffin, 1:1:1 (w/w)). Microtubules were visualized by use of a video-enhanced microscope system consisting of a Zeiss standard research microscope with a $\times 100$ (NA 1.25) objective, $\times 2$ optovar, a wide-band-pass green interference filter (wavelength 546 nm), and standard differential interference contrast (DIC) optics. The microscope was equipped with a 100-W mercury arc lamp set at critical illumination, and was connected to a MTI Series 68 Newvicon video camera equipped with remote gain and black offset level, with the video output directed to a RCA monochrome monitor¹⁰. The velocity of microtubules was measured in real time using computer-assisted analysis. The video output of an Amiga 1000 computer was mixed with the output of the MTI video camera using a video mixer installed by DAGE Inc. allowing the image of the computer cursor (an arrow) to be superimposed on the image of the microtubules. The position of the computer cursor was controlled by the movement of a hand-operated mouse. A computer program was written which caused the *x* and *y* coordinates of the cursor, as well as the time measured by an internal clock in the computer, to be recorded by the computer when the input button on the mouse was pressed. In practice, the cursor would be moved to the site of a microtubule end and the mouse button triggered to mark its position. After an arbitrary period (usually 10–30 s) the cursor would be moved to the new site of the same microtubule end and again the mouse button triggered. The computer would then calculate the distance between the two *x*, *y* coordinates (using scaling factors between *x*, *y* coordinates and actual distances measured previously with a stage micrometer) and then divide it by the time difference to obtain the velocity of the microtubule. After the measurement of an arbitrary number of microtubules (usually 15–25) the data collection would be stopped and the computer would calculate the mean velocity and standard deviation, as well as print out a histogram of microtubule velocities.



ATPase exhibit similar requirements for divalent cations (Mg^{2+}) in conjunction with the hydrolysable nucleotide (ATP); and third, both assays are sensitive to the same inhibitors.

Several properties of the kinesin-microtubule system are shared with the actin-myosin and dynein-microtubule systems⁴. For example, kinesin binds to microtubules in the absence of nucleotides (refs 5, 7, 8 and unpublished observations), kinesin is dissociated from microtubules by Mg-ATP (refs 5, 7 and unpublished observations), and the Mg^{2+} -ATPase activity of kinesin is activated by microtubules. These observations suggest that these three major eukaryotic cell motility systems (actomyosin, microtubule-dynein, microtubule-kinesin) may operate through similar mechanochemical pathways.

The biological functions of sea urchin egg kinesin remain unknown. Echinoderm eggs and early embryos undergo a variety

of microtubule-based motile events, including vesicle/organelle translocation, pronuclear migration and mitosis. Clearly egg kinesin, possibly acting in concert with other microtubule-based ATPases^{19–22}, may mediate some of these movements.

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- Gibbons, B. H. & Gibbons, I. R. *J. Cell Biol.* **54**, 75–97 (1972).
- Brokaw, C. J. & Benedict, B. *Archs Biochem. Biophys.* **125**, 770–778 (1968).
- Gibbons, I. R. *J. Cell Biol.* **91**, 107s–124s (1981).
- Johnson, K. A. *Rev. Biophys. biophys. Chem.* **14**, 161–188 (1985).
- Vale, R. D., Reese, T. S. & Sheetz, M. P. *Cell* **42**, 39–50 (1985).
- Vale, R. D., Scholey, J. M. & Sheetz, M. P. *Trends biochem. Sci.* **11**, 464–468 (1986).
- Vale, R. D., Schnapp, B. J., Sheetz, M. P. & Reese, T. S. *J. Cell Biol.* **103**, 552a (1986).
- Scholey, J. M., Porter, M. E., Grissom, P. M. & McIntosh, J. R. *Nature* **318**, 483–486 (1985).
- Schnapp, B. J., Kahn, S., Sheetz, M. P., Vale, R. D. & Reese, T. S. *J. Cell Biol.* **103**, 551a (1986).
- Porter, M. E. *et al. J. Biol. Chem.* **262**, 2794–2802 (1987).
- Kuznetsov, S. A. & Gelfand, V. I. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8530–8534 (1986).
- Bloom, G. S., Wagner, M. C. & Brady, S. T. *J. Biol. Chem.* (submitted).
- Adelstein, R. S., Pato, M. D., Sellers, J. R., de Lanerolle, P. & Conti, M. A. in *Basic Biology of Muscles: A Comparative Approach* (eds Twarog, B. M., Levine, R. J. C. & Dewey, M. M.) 273–281 (Raven, New York, 1982).

- Sellers, J. R., Spudich, J. A. & Sheetz, M. P. *J. Cell Biol.* **101**, 1897–1902 (1985).
- Suprenant, K. A. & Marsh, J. C. *J. Cell Sci.* **87**, 71–84 (1987).
- Hayashi, M. *Archs Biochem. Biophys.* **165**, 288–296 (1974).
- Brady, S. T. *Nature* **317**, 73–75 (1985).
- Penningroth, S. M., Rose, P. M. & Peterson, D. D. *J. Cell Biol.* **103**, 552a (1986).
- Scholey, J. M., Neighbors, B., McIntosh, J. R. & Salmon, E. D. *J. Biol. Chem.* **259**, 6516–6525 (1984).
- Dinenberg, A. S., McIntosh, J. R. & Scholey, J. M. *Ann. N. Y. Acad. Sci.* **466**, 431–435 (1986).
- Pratt, M. M. *Int. Rev. Cytol.* **87**, 83–105 (1984).
- Collins, C. A. & Vallee, R. B. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4799–4803 (1986).
- Williams, R. C. Jr & Lee, J. C. *Meth. Enzym.* **85**, 376–385 (1982).
- Amos, L. A. *J. Cell Sci.* **87**, 105–111 (1987).
- Vallee, R. B. & Bloom, G. S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6259–6263 (1983).
- Seals, J. R., McDonald, J. M., Bruns, D. & Jarett, L. *Analyt. Biochem.* **90**, 785–795 (1978).
- Goodno, C. C. *Meth. Enzym.* **85**, 116–123 (1982).

Evidence for the involvement of ATP in co-translational protein translocation

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Identification of the source of energy for protein translocation across biological membranes is important in understanding the mechanism of this process. In eukaryotic cells, the tight coupling between translation and translocation¹ and firm attachment of the secreting ribosomes to membranes², as well as theoretical calculations³, have led to the suggestion that energy derived from protein synthesis is sufficient for protein translocation. On the other hand, in bacterial systems neither the attachment of ribosomes to membrane (other than nascent chains)⁴ nor tight coupling of translocation to translocation has been observed⁵⁻⁹. Moreover, certain proteins can be translocated across membranes either at the time of, or after, translation^{7,8,10}. The separation of protein translocation from translation has made possible the demonstration that ATP hydrolysis is essential for post-translational protein translocation across bacterial membranes¹¹⁻¹⁴ and, more recently, also across canine and yeast endoplasmic reticulum membranes¹⁵⁻¹⁹. Here we report that certain ATP analogues inhibit co-translational protein translocation at concentrations that do not interfere with protein synthesis, suggesting that ATP is also required for co-translational protein translocation.

The requirement for ATP, first demonstrated in chloroplasts²⁰, could be due to a unique aspect of post-translational translocation not associated with co-translational translocation, such as ATP-dependent unfolding or conformational change of already synthesized precursor molecules, so that they become or remain competent for translocation^{8,21,22}. Another possibility is that post-translational translocation might use an entirely different pathway of protein secretion. On the other hand, ATP hydrolysis could be involved in membrane functions required for all protein transport across membranes and therefore could be a general requirement for protein translocation.

A direct demonstration that ATP is required for co-translational translocation is difficult, because ATP is also required for protein synthesis as a cofactor in the amino-acylation of transfer RNAs. To circumvent this difficulty, we attempted to use instead a specific inhibitor of translocation based on our earlier findings¹³ that post-translational protein translocation across *Escherichia coli* membranes involves an ATP binding site and is inhibited by several ATP analogues that do not interfere with protein synthesis. Among several analogues tested, 2',3'-cyclic AMP had no effect on protein synthesis up to 10 mM (Fig. 1a), but at 2.5 mM it severely inhibited the operationally co-translational translocation of precursors of alkaline phosphatase and *OmpA* protein (Fig. 1b). Most protein translocation occurred co-translationally in such *in vitro* systems. First, bacterial proteins can be efficiently translocated either co- or post-translationally^{7,8} and indeed alkaline phosphatase was mostly secreted co-translationally in cells, being synthesized on membrane-bound polysomes^{4,7}. When membranes were available to interact with secreting nascent peptides¹⁰ at least some co-translational translocation should have taken place. Second, because maximal translation and translocation had occurred after ~15 min *in vitro*⁷, the amount of post-translational translocation was only about 40% of the amount of co-translational translocation in the system used, where the total reaction time was 18 min or less. Finally, analysis of the association of nascent peptides with

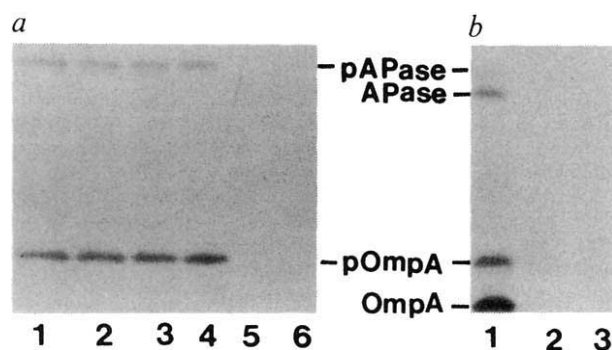


Fig. 1 Effects of ATP analogues on protein synthesis and protein translocation. *a*, The reaction mixture, in 0.1 ml, contained 50 mM Tris-HCl (pH 7.6), 40 mM KCl, 20 mM NH₄Cl, 1 mM Tris-ATP, 5 mM phosphoenolpyruvate-Tris, 3 µg pyruvate kinase, 0.02 mM GTP-Tris, 6.5 mM magnesium acetate, 1 mM spermidine-HCl, 8 mM putrescine-HCl, 2 A₂₆₀ units of S30 extract, 2 mM dithiothreitol, appropriate mRNA, 20 µCi of ³⁵S-methionine (1,000 Ci mmol⁻¹), and 19 other amino acids at 0.05 mM each. Protein synthesis was carried out at 40 °C for 15 min as previously described⁸. Lane 1, no other addition; lanes 2-4, with 2.5, 5, 10 mM 2',3'-cyclic AMP, respectively; lane 5, with 2.5 mM triphosphophate; lane 6, with 2.5 mM ATPγS. The samples were subjected to electrophoresis in a SDS-acrylamide gel, and the gel was fluorographed as described⁸. *b*, Protein synthesis, with or without cAMP, corresponding to lanes 1-3 in *a*, was carried out at 40 °C for 3 min (to minimize the inhibitory effect of membranes⁸), membrane vesicles from *E. coli* K12 strain D10 were added, and the co-translational translocation was continued for 15 min (ref. 8). The mixtures were treated with pronase, the membranes were isolated and the translocated products were analysed as described⁸. Abbreviations: pAPase, alkaline phosphatase precursor; APase, alkaline phosphatase; pOmpA, precursor of *OmpA* protein.

Table 1 Distribution of peptides in various fractions during translation

Conditions	Proportion of peptides in each fraction (%)			
	Free polysome	Membrane-associated ribosome	Free membrane	Free peptides
Translation, 5 min				
-Puromycin	21.1	28.9	19.4	30.6
+Puromycin	2.7	3.7	34.1	59.5

Protein synthesis was carried out with ³⁵S-methionine as in Fig. 1 except membranes were added 2 min after translation was started and translation/translocation was continued for an additional 5 min. To half the reaction mixture was added chloramphenicol (to 100 µg ml⁻¹) and this mixture cooled on ice; the other half was treated with puromycin (to 100 µg ml⁻¹) for 1 min before addition of chloramphenicol and cooling. Each portion was layered on a block gradient containing 0.8 M/1.3 M/1.8 M sucrose in 10 mM Tris-HCl, pH 7.6, 50 mM KCl and 10 mM magnesium acetate and centrifuged in a Spinco SW50.1 rotor at 42,000 r.p.m. for 18 h at 4 °C. Fractions were collected and assayed for hot trichloroacetic-acid-precipitable radioactive counts. The pellet fraction was taken to be nascent peptides associated with free polysomes, fractions at the interface of 1.3 and 1.8 M sucrose to contain nascent peptides present in membrane-associated polysomes, those at that of 0.8 M and 1.3 M sucrose to be free membranes, and top fractions to be free peptides.

membrane vesicles in sucrose gradients⁴ showed that about half of the nascent peptides were associated with membrane-bound polysomes after 5 min incubation to allow translocation in a system where not all peptides were destined to be secreted, and a large fraction was released into membranes by puromycin, indicating that much translocation occurred co-translationally (Table 1). The complete inhibition by 2',3'-cyclic AMP in these

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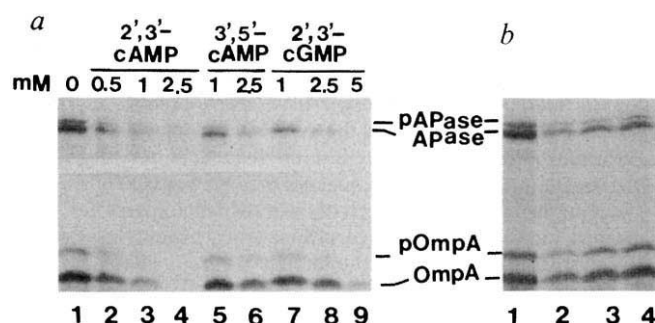


Fig. 2 Inhibition of co-translational translocation by cyclic nucleotides and reversal by regeneration of ATP. *a*, Co-translational translocation was carried out, as in Fig. 1*b*, with various concentrations of 2',3'-cAMP, 3',5'-cAMP, or 2',3'-cGMP, at which little effect on protein synthesis was observed. Far left lane, control without the analogues. *b*, Translation and translocation were carried out as in Fig. 1*a*, except that 10 mM creatine phosphate and creatine-phosphate kinase were used to replace phosphoenolpyruvate kinase, without (lane 1) or with (lane 2) 1.5 mM 2',3'-cAMP or with 1.5 mM 2',3'-cAMP plus 4 mM (lane 3) or 6 mM (lane 4) creatine phosphate. Abbreviations as in Fig. 1.

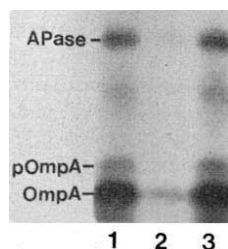


Fig. 3 Inactivation of membranes for protein translocation by photoaffinity labelling with azido-ATP. Membranes, with nucleotides as indicated, were irradiated with long-wavelength ultraviolet radiation (at 366 nm), diluted with buffer, isolated and used for co-translational translocation as in Fig. 1*b*. Lane 1, control with 3 mM ATP; lane 2, with 1 mM 8-azido-ATP; lane 3, with 1 mM 8-azido-ATP and 5 mM ATP. Abbreviations as in Fig. 1.

conditions (Fig. 1*b*) therefore showed that this nucleotide also inhibited co-translational translocation.

At similar concentrations, 3',5'-cAMP or 2',3'-cGMP also inhibited the translocation without interfering with translation, though the inhibition was less than with 2',3'-cAMP (Fig. 2*a*). Similarly, at lower concentrations (<1.5 mM) that did not interfere with protein synthesis, 5'-AMP, though not as inhibitory as cyclic 2',3'-AMP, was a much better inhibitor than 2'-AMP or 3'-AMP for co-translational translocation (data not shown). Why 2',3'-cAMP is more effective as an inhibitor of co-translational translocation than 3',5'-cAMP or other nucleotides is not clear; it is possible that the former is metabolically more stable in *E. coli* extracts or is more related to the ATP hydrolysis product during translocation. The broad specificity of the inhibiting effect indicated that it was not a specific 3',5'-cAMP-mediated process.

To determine whether the action of 2',3'-cAMP is due to competition for an ATP binding site which functions in translocation, we attempted to reverse the inhibition by supplying additional ATP. The direct addition of magnesium ATP (4–5 mM) to the translating system inhibited protein synthesis (data not shown). However, we found that accelerating the regeneration of ATP by supplementing phosphoenolpyruvate (4 mM, which slightly inhibited the synthesis of alkaline phosphatase precursor; data not shown) or by addition of creatine phosphate

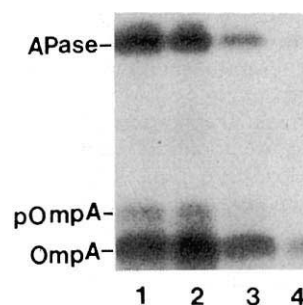


Fig. 4 Inhibition of co-translational translocation by 2',3'-cAMP with membranes deleted with H^+ -ATPase. The translocation was carried out as in Fig. 1*b*, except membrane vesicles of CK1801 (ΔH^+ -ATPase)¹² were used. Lane 1, control with no cAMP; lanes 2–4, with 0.5, 1, 2 mM 2',3'-cAMP respectively. Abbreviations as in Fig. 1.

(4–6 mM, plus its kinase; Fig. 2*b*) partially relieved the inhibition of translocation by 1.5 mM 2',3'-cAMP. At this concentration inhibition of translocation is not complete: it was used to observe the reversal effect better. Indeed, the use of creatine phosphate and creatine-phosphate kinase in the translation and translocation system without the ATP analogue provided independent evidence that ATP is also required for co-translational translocation: increasing concentrations of creatine phosphate (up to 12 mM) had little effect on protein translation, but greatly enhanced the translocation of precursors of alkaline phosphatase and the *OmpA* protein (data not shown).

Additional evidence for a function of ATP in co-translational translocation came from photoaffinity labelling with ATP analogues. We had found earlier that membranes lost their ability to mediate post-translational translocation when subjected to photoreaction in the presence of azido-ATP or azido-AMP¹³. The specificity of this process was confirmed by the observation that membrane inactivation was prevented by ATP or AMP¹³. We have now found that photoaffinity labelling of membranes with azido-ATP also renders them unable to mediate co-translational protein translocation and that the inactivation is prevented by ATP (Fig. 3). These observations imply that a functional ATP binding site on the cytoplasmic membrane is required both for post-translational and co-translational protein translocation.

To test the possibility that cyclic AMP may be acting on certain functions of H^+ -ATPase in membranes, thereby affecting translocation indirectly by interfering with protonmotive force to disturb membrane structures^{11,12,14,23}, we used membranes prepared from a mutant with a deletion of all genes for H^+ -ATPase¹². Co-translational protein translocation across these membranes was still severely inhibited by 2',3'-cAMP (Fig. 4), indicating that the action of 2',3'-cAMP was probably not related to the functions of H^+ -ATPase.

Our results suggest that ATP is required not only for post-translational protein translocation but also for co-translational translocation. They support the notion that ATP hydrolysis is involved in a general aspect of protein translocation across the membranes, whether co- or post-translational. This general element seems to involve an ATP binding site in the membrane¹³, at which the ATP analogues compete for ATP causing inhibition. One possibility is that ATP hydrolysis is used to drive the peptides across the membranes. Another possibility is that ATP hydrolysis is necessary for the phosphorylation of specific membrane proteins that are constituents of the secretory pathway. The energy or the modulation of phosphorylation and dephosphorylation of these proteins could trigger certain membrane topological or conformational changes, which could also be partially brought about by the protonmotive force^{11,12,14}, thereby permitting the initial interaction of the translocating protein

precursor with a putative membrane receptor or its passage through the secretory channel in the membrane. Indeed, we have observed that several membrane proteins are phosphorylated by [γ - 32 P]ATP, but not by [γ - 35 S]ATP, an analogue which cannot support protein translocation^{11,13}, and that the phosphorylated proteins were rapidly dephosphorylated (unpublished data). The correlation of phosphorylation-dephosphorylation with bacterial protein translocation would be an important step toward our understanding of the mechanisms of protein translocation. This would mean that a reaction common to co- and post-translational translocation involves ATP hydrolysis, presumably phosphorylation and dephosphorylation of certain membrane proteins that facilitate the translocation of proteins across membranes.

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- Walter, P. & Blobel, G. *J. Cell Biol.* **91**, 557-561 (1981).
- Sabatini, D. D., Kreibich, G., Morimoto, T. & Adesnik, M. *J. Cell Biol.* **92**, 1-22 (1982).
- Von Heijne, G. & Blomberg, C. *Eur. J. Biochem.* **97**, 175-181 (1979).
- Smith, W. P., Tai, P. C. & Davis, B. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 814-817 (1978).
- Davis, B. D. & Tai, P. C. *Nature* **283**, 433-437 (1980).
- Wickner, W. *Science* **210**, 861-868 (1980).
- Randall, L. L. & Hardy, S. J. S. *Microbiol. Rev.* **48**, 290-298 (1984).
- Chen, L., Rhoads, R. & Tai, P. C. *J. Bact.* **161**, 973-980 (1985).
- Muller, M. & Blobel, G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7421-7425 (1984).
- Tai, P. C. *Curr. Top. Microbiol. Immun.* **125**, 43-58 (1986).
- Chen, L. & Tai, P. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4384-4388 (1985).
- Chen, L. & Tai, P. C. *J. Bact.* **167**, 389-392 (1986).
- Chen, L. & Tai, P. C. *J. Bact.* **168**, 828-832 (1986).
- Geller, B., Movva, N. R. & Wickner, W. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4219-4222 (1986).
- Perara, E., Rothman, R. E. & Lingappa, V. R. *Science* **232**, 348-352 (1986).
- Caulfield, M. P., Duong, L. T. & Rosenblatt, M. *J. Biol. Chem.* **261**, 10953-10956 (1986).
- Waters, M. G. & Blobel, G. *J. Cell Biol.* **102**, 1543-1550 (1986).
- Rothblatt, J. & Meyer, D. *EMBO J.* **5**, 1031-1036 (1986).
- Hansen, W., Garcia, P. C. & Walter, P. *Cell* **45**, 397-406 (1986).
- Grossman, A., Bartlett, S. & Chua, N. H. *Nature* **285**, 625-628 (1980).
- Eilers, M. & Schatz, G. *Nature* **322**, 228-232 (1986).
- Randall, L. L. & Hardy, S. J. S. *Cell* **46**, 921-928 (1986).
- Bakker, E. P. & Randall, L. L. *EMBO J.* **3**, 895-900 (1984).

The mapping of a cDNA from the human X-linked Duchenne muscular dystrophy gene to the mouse X chromosome

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The recent discovery of sequences at the site of the Duchenne muscular dystrophy (DMD) gene in humans^{1,2} has opened up the possibility of a detailed molecular analysis of the genes in humans and in related mammalian species. Until relatively recently, there was no obvious mouse model of this genetic disease for the development of therapeutic strategies. The identification of a mouse X-linked mutant showing muscular dystrophy³, *mdx*, has provided a candidate mouse genetic homologue to the DMD locus; the relatively mild pathological features of *mdx* suggest it may have more in common with mutations of the Becker muscular dystrophy type¹ at the same human locus, however. But the close genetic linkage of *mdx* to *G6PD* and *Hprt* on the mouse X chromosome⁴⁻⁶, coupled with its comparatively mild pathology, have suggested that

the *mdx* mutation may instead correspond to Emery Dreifuss muscular dystrophy which itself is closely linked to DNA markers at Xq28-qter in the region of *G6PD* on the human X chromosome⁷. Using an interspecific mouse *domesticus*/*spretus* cross⁸, segregating for a variety of markers on the mouse X chromosome, we have positioned on the mouse X chromosome sequences homologous to a DMD cDNA clone. These sequences map provocatively close to the *mdx* mutation and unexpectedly distant from sparse fur, *spf*, the mouse homologue of OTC (ornithine transcarbamylase) which is closely linked to DMD on the human X chromosome⁹.

An interspecific *Mus domesticus*/*Mus spretus* cross was initiated for the mapping of molecular markers to the mouse X chromosome (Fig. 1). A female *M. domesticus* mouse carrying the X-linked coat-texture mutations Harlequin (*Hq*), Tabby (*Ta*) and Lined (*Li*) was crossed to a male *M. spretus*. Four female progeny, two carrying *Hq* and two carrying *Ta*, were backcrossed to inbred 129 mice and a total of 232 progeny were produced segregating for either *Hq* or *Ta*. A variety of random DNA markers isolated by microcloning of the mouse X chromosome¹⁰ and gene probes have been mapped and ordered on the mouse X chromosome using recombinational and pedigree analysis (N.B., E.M.C.F., J.S.C., M.F.L. and S.D.M.B., manuscript in preparation). The crosses take advantage of the high level of restriction fragment length variation between the diverged *domesticus* and *spretus* genomes⁸. Figure 1 illustrates the position of pertinent loci mapped in the cross and used for the positional assignment of mouse sequences homologous to a cDNA from the human DMD gene.

The human adult muscle cDNA sequence used in these experiments (pCA1) is 1.8 kilobases (kb) long and maps in the Xp21 region of the human X chromosome (G.S.C., Y. Edwards and K.E.D., manuscript in preparation). The exons detected by this cDNA cover at least 100 kb of human genomic DNA and they are deleted in several DMD patients. No major autosomal cross-hybridizing bands are seen in Southern blots; all the bands map to the Duchenne gene region.

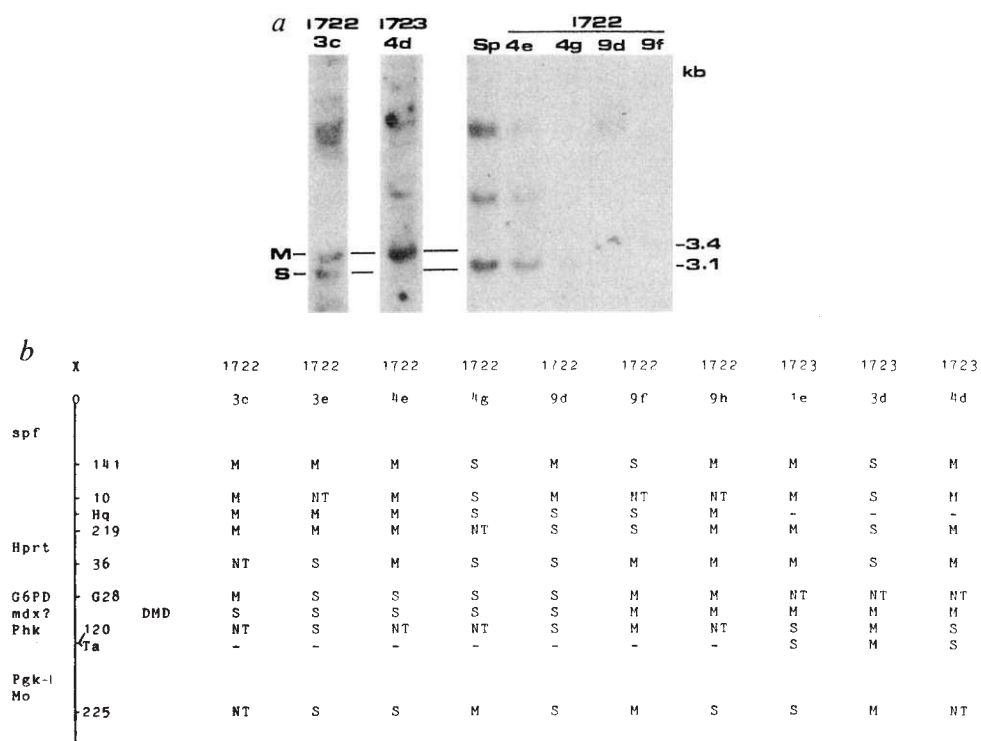
A variety of interspecific mouse backcross progeny were hybridized to pCA1 (Fig. 1) and scored for the presence of a *spretus* (S) or *domesticus* (M) allele. Hybridization of the pCA1 probe to progeny DNAs reveals a *domesticus* allele, M (Fig. 1a) at 3.4 kb and a *spretus* allele, S (Fig. 1a) at 3.1 kb. A variety of fainter bands appear to segregate either with the M or S allele and, in addition, a number of other faint bands were held in common between *domesticus* and *spretus*. Both the pedigree analysis (Fig. 1b) and recombinational analysis (Table 1) indicate that pCA1 maps to the interval between G28 and *Ta*. For example, in the pedigree analysis (Fig. 1b), mouse 1723.4d indicates that DMD maps proximal to 120 and *Ta*; however, mouse 1722.3c indicates that DMD maps distal to G28. The recombinational analysis (Table 1) agrees with this positioning. For example, the DMD-10 genetic distance is 19 cM and the DMD-*Hq* distance is 20 cM, whereas G28, localized some 14 cM distal to *Hq*, is 5 cM from DMD. Thus DMD maps proximal and close to *Ta*; the DMD-*Ta* recombination distance is 6 cM.

In mouse, *mdx* is known to map 10 cM proximal to the Mottled (*Mo*) locus⁵ and ~12 cM proximal to *Pgk-1* (ref. 4) and therefore ~6-9 cM proximal to *Ta*. *Mdx* must also be relatively closely linked to *G6PD*^{6,11}. But no information is available on direct *G6PD*-*mdx* recombinational distances from mouse crosses segregating for both *G6PD* and *mdx* alleles together and it cannot, at this point, be stated whether *mdx* maps to a position that is proximal or distal to *G6PD*. If the position is proximal, it would lie within the *G6PD*-*Hprt* linkage groups apparently conserved between mice and humans¹¹ and may correspond to Emery Dreifuss muscular dystrophy. But if *mdx* is found to be distal to *G6PD*, the close linkage of *mdx* to the DMD-homologous sequences on the mouse X chromosome would add weight to the suggestion that *mdx* is homologous to the DMD locus. Given the large size of the DMD gene, it will require very refined genetic analysis to determine the identity

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Fig. 1 Segregation of DMD homologous sequences in mouse interspecific *domesticus/spretus* crosses and pedigree analysis. **a**, Segregation of the *domesticus* (M) and *spretus* (S) alleles in a number of backcross progeny from mouse interspecific crosses segregating for *Hq* or *Ta*. *Msp*I-digested progeny DNAs and *Mus spretus* DNA (lane marked Sp) were hybridized to the radiolabelled pCA1 probe. A variety of fainter bands detected are constant between *domesticus* and *spretus* mice. **b**, Pedigree analysis of the *Mus domesticus/Mus spretus* interspecific cross. A variety of DNA markers (indicated to the right of the X chromosome) have been mapped in the interspecific crosses segregating for *Hq* or *Ta*. All markers, except G28 (ref. 11), were isolated by microdissection and microcloning of the mouse X chromosome¹⁰. Clone G28 is a mouse homologue of the human pGD3 clone mapping to the 3' side of the human *G6PD* gene. The genotypes of several backcross progeny are indicated at various loci. In the case of female mice the genotype of the X chromosome derived from the female parent is indicated. For each mouse the DMD genotype was also determined and its position on the X chromosome consistent with the pedigree analysis indicated. The position of several other loci not segregating in the analysis is indicated to the left of the X chromosome. NT, not tested.



For each mouse the DMD genotype was also determined and its position on the X chromosome consistent with the pedigree analysis indicated. The position of several other loci not segregating in the analysis is indicated to the left of the X chromosome. NT, not tested.

Methods. A female *Mus domesticus* mouse carrying the X-linked coat-texture mutations Harlequin (*Hq*), Tabby (*Ta*) and Lined (*Li*) (genotype: *Hq + Li + Ta +*) was crossed to a male *Mus spretus*. Four female progeny, two carrying *Hq* and two carrying *Ta*, were backcrossed to male 129 inbred mice. A total of 232 progeny were produced. Tail DNAs¹⁴ from a number of progeny segregating for either *Hq* or *Ta* were digested with *Msp*I or *Taq*I, fractionated on 0.8% agarose gels, blotted to Hybond-N (Amersham) and probed with hexamer-labelled¹⁵ pCA1 and a number of other molecular markers to the mouse X chromosome¹⁰. Hybridization of the pCA1 probe was carried out at 42 °C in 3 × SSC, 50% formamide, 0.5% SDS. Filters were washed in 3 × SSC at 65 °C. Hybridization of other probes (141, 10, 219, 36, G28, 120 and 225) were carried out at 65 °C in 6 × SSC, 0.5% SDS, 10% Dextran Sulphate and filters washed in 1 × SSC at 65 °C.

Table 1 Recombinational analysis of a variety of X-chromosome markers

	10	Hq	219	36	G28	DMD	120	Ta	225
141	7/164 4 ± 2	13/142 9 ± 2	12/92 13 ± 4	5/54 9 ± 4	20/87 23 ± 5	21/74 28 ± 5	22/67 33 ± 6	19/63 30 ± 6	24/45 53 ± 7
10		3/127 2 ± 1	2/73 3 ± 2	1/41 2 ± 2	11/77 14 ± 4	12/65 19 ± 5	15/49 31 ± 7	14/46 30 ± 7	14/33 42 ± 9
Hq			1/50 2 ± 2	2/26 8 ± 5	9/65 14 ± 4	8/41 20 ± 6	5/23 22 ± 9	—	12/26 46 ± 10
219				2/44 5 ± 3	12/60 20 ± 5	15/57 26 ± 6	21/69 30 ± 6	15/46 33 ± 7	21/44 48 ± 8
36					5/29 17 ± 7	7/33 21 ± 7	8/27 30 ± 9	7/28 25 ± 8	19/44 43 ± 8
G28						3/61 5 ± 3	5/39 13 ± 5	4/25 16 ± 7	9/28 32 ± 9
DMD							4/39 10 ± 5	2/35 6 ± 4	6/25 24 ± 9
120								2/48 4 ± 3	5/27 19 ± 8
Ta									4/19 21 ± 9

Progeny from a *Mus domesticus/Mus spretus* interspecific cross were analysed. Upper value, number of recombinants out of the total number of mice analysed for each two-point analysis; lower value, the calculated genetic distance in centimorgans with standard error.

of *mdx* and DMD as crossovers detected between *mdx* and the DMD probe would not necessarily rule out homology and may represent crossovers within the gene itself. In any case, the position of the DMD sequences on the mouse X chromosome is very provocative because, if DMD is not homologous to *mdx*, then a cluster of muscle-related genes apparently exists at this region of the mouse X chromosome—*mdx*, DMD and *Phk* (the locus which controls phosphorylase kinase activity in

skeletal muscle) (see Fig. 1).

Finally, the data enhance our knowledge of the comparative evolution of the mouse and human X chromosomes. DMD and OTC are closely linked on the human X⁹ but we show here that *spf* (OTC mouse homologue) and mouse DMD sequences lie far apart on the mouse X chromosome. This clearly indicates that one of the major ancestral breakpoints that have been postulated to explain the evolution of mouse and human X

chromosomes^{12,13} can be found between the DMD and OTC loci on the human X.

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1. Monaco, A. P. & Kunkel, L. M. *Trends Genet.* **3**, 33-37 (1987).
2. Smith, T. J. *et al. Nucleic Acids Res.* **15**, 2167-2174 (1987).
3. Bulfield, G., Siller, W. G., Wight, P. A. L. & Moore, K. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1189-1192 (1984).
4. Chapman, V. M., Murawski, M., Miller, D. & Swiatek, D. *Mouse News Lett.* **72**, 120 (1985).
5. Bulfield, G. & Isaacson, J. H. *Mouse News Lett.* **72**, 97 (1985).
6. Peters, J. & Ball, S. T. *Mouse News Lett.* **72**, 17-18 (1985).
7. Hodgson, S. *et al. Hum. Genet.* **74**, 409-416 (1986).
8. Brown, S. D. M. *Trends Genet.* **1**, 219-220 (1985).
9. Davies, K. E. *et al. Nucleic Acids Res.* **13**, 3419-3426 (1985).
10. Fisher, E. M. C., Cavanna, J. S. & Brown, S. D. M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5846-5849 (1985).
11. Avner, P., Amar, L., Arnaud, D., Hanauer, A. & Cambron, J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
12. Buckle, V. J. *et al. Clin. Genet.* **25**, 275-285 (1984).
13. Buckle, V. J. *et al. Human Gene Mapping* **8**, 594-595 (1985).
14. Grosschedl, R., Weaver, D., Baltimore, D. & Constantini, F. *Cell* **38**, 647-658 (1984).
15. Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **137**, 266-267 (1984).

Localization of the region homologous to the Duchenne muscular dystrophy locus on the mouse X chromosome

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Recent progress has resulted in part of the gene mutated in Duchenne and the milder Becker muscular dystrophies being cloned¹⁻³ and has suggested that the gene itself extends over 1,000 to 2,000 kilobases (kb) (ref. 4). To study how mutations in this gene affect muscle development and integrity, it would be of interest to have available a mouse model of the human disease. The mouse *mdx* mutation affects muscle and confers a mild dystrophic syndrome, but it is not clear whether this mutation is equivalent to Duchenne/Becker muscular dystrophy in man⁵. Here we describe the use of two sequences from the human Duchenne muscular dystrophy (DMD) gene that cross-hybridize to mouse X-linked sequences to localize the gene homologous to DMD in the mouse. Both sequences map to the region of 10 centimorgan lying between the Tabby (*Ta*) and *St14-1* (*DxPas8*) loci, close to the phosphorylase b kinase locus (*Phk*). By analogy with the human X-chromosome, we conclude that the region in the mouse around the *G6pd* and *St14-1* loci may contain two genes corresponding to distinct human myopathies: Emery Dreifuss muscular dystrophy which is known to be closely linked to *St14-1* in man^{6,7} and the DMD homologue described here.

The two conserved sequences from the Duchenne gene used as probes for the mouse genome were E87-25 and CCR1. E87-25 corresponds to an exon sequence in the Pert87 region identified by L. Kunkel and his colleagues². CCR1 was isolated from a cosmid walk around the X-21 translocation breakpoint characterized by Worton *et al.*⁸. This particular sub-clone was selected

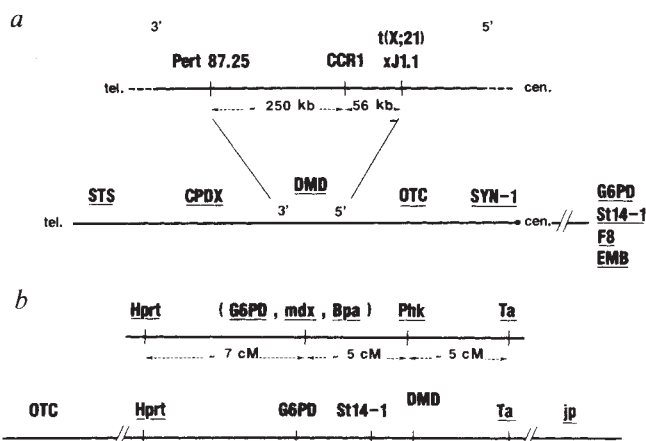


Fig. 1 *a*, Representation of the human Xp21 DMD/BMD locus. The different levels show the organisation of the region and its orientation with different degrees of detail. The E87-25 probe corresponds to the Pert87-25 exonic sequence² and was re-cloned using two 41mer oligonucleotides complementary over a 13 nucleotide region according to the published sequence. After annealing and infilling with the Klenow fragment of DNA polymerase, the oligonucleotides were ligated into the *Sma*I site of a pBS-M13(+) Blue Scribe vector. CCR1 is a 4.9-kb *Pst*I fragment isolated from a cosmid and subcloned into the *Sma*I site of a pBS-M13(+) Blue Scribe vector (R.H., in preparation). A 1,000 base pair (bp) CCR1 *Hpa*I-*Bst*NI fragment and a 450-bp *Pvu*II fragment of E87-25 were used for nick translation. *b*, Representation of the region of the mouse X-chromosome containing the DMD homologue. The upper line diagram shows the position of various known mutants within this region and the lower line diagram the equivalent position of the various molecular markers tested.

on the basis of its cross-hybridization to mouse, hamster, rat and chicken genome DNAs and has been shown to be X-linked in both the hamster and mouse (R.H., unpublished data and see below). The two probes are separated by a distance of about 250 kilobases (kb) in man, CCR1 being on the 5' side (Fig. 1).

Use of a panel of mouse-hamster somatic cell hybrids, containing mouse X chromosomes deleted to various extents⁹ has confirmed that both CCR1 and E87-25 detect sequences present on the mouse X chromosome (Fig. 2A lanes *a*, *b* and *g*, *h*) with no evidence for the presence of cross-reacting sequences on other chromosomes and shown that both sequences must be localized proximally to the T16H X-chromosome breakpoint ~4 centimorgans (cM) proximal to *Ta* (ref. 10). (One centimorgan corresponds to roughly 1% recombination.) This is based on the retention of the hybridization signals on all the hybrids tested including B20c12 (Fig. 2A, tracks *d-f*) which carries a translocated X:16 chromosome and lacks the distal part of the mouse X chromosome including the Tabby (*Ta*) locus.

Finding a *Taq*I restriction fragment length polymorphism (RFLP) between the B6CBARI strain belonging to *Mus musculus domesticus* and the inbred SPE/Pas strain derived from *Mus spretus*, for both the CCR1 and E87-25 probes, made possible the use of an interspecies backcross between these two strains (Fig. 2B) to localize more precisely the mouse homologue of the DMD-related sequences. Meiotic recombinants from this backcross had previously been typed for a large number of X-linked loci including genes for ornithine transcarbamylase (*Otc*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), glucose-6-phosphate dehydrogenase (*G6pd*), proteolipid protein (*Plp* same as jimpy) as well as the Tabby (*Ta*) gene, a series of loci detected by anonymous X-chromosome-specific murine probes and a large number of cross-reacting human probes including probes for coagulation factors VIII and IX and anonymous sequences *St14-1* and M2C (refs 11 and 14, and unpublished data). The localization of the markers most

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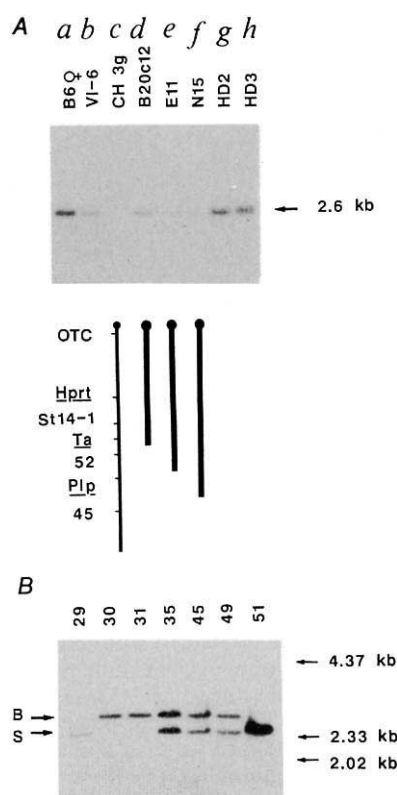


Fig. 2 A, Hybridization pattern of the various cell lines with probe CCR1 on *TaqI* restricted DNAs. Lanes a, g and h, female mouse DNAs; lane b, VI-6, a Chinese hamster/mouse hybrid containing mouse chromosomes X and 16; lane c, Chinese hamster control CH3g, lanes d-f, Chinese hamster/mouse hybrid cell lines containing deleted mouse X chromosomes derived from the T16H, T14R1 and T6R1 mouse translocations⁹. Below, the extent of the X chromosome present in each of these hybrids as identified by marker studies. (For details see ref. 9). B, Hybridization of the CCR1 probe to *TaqI* restricted backcross animal DNAs. Numbers identify individual backcross animals. Hybridizations were carried out at 42 °C in 40% formamide, 5% dextran sulphate. Wash conditions were 0.5 × SSC, 0.05% SDS at 60 °C for CCR1, and 3 × SSC, 0.05% SDS at 60 °C for E87-25.

relevant to this study is shown in Fig. 3. CCR1 showed 12.5% recombination with *Hprt*, 4.6% with *G6pd* and 5.8% with *Ta* (10/80, 3/65 and 4/68 progenies studied). Recombination frequencies were similar with E87-25 (10.2%, 4.6% and 7.7%) with *Hprt*, *G6pd* and *Ta* respectively. Given the localization of markers in this region, this suggests that the probes lie distal to *G6pd* (*Gdx*).

Confirmation of this localization was obtained by pedigree analysis allowing correlation of the cross-over events that have occurred on the X chromosome of each backcross progeny with the allelic distribution detected by particular probes¹²⁻¹⁴. Illustrative pedigrees for some of the informative animals are shown in Fig. 3 for both the CCR1 and E87-25 probes. The illustrated animals define the CCR1 and E87-25 regions as proximal to *Ta* (animals 21, 29, 52 and 84) and distal to *St14-1* (*DXPas8*) (animals 18 and 38).

Extrapolation of the results of the recombination analysis to the pre-existing genetic map of the mouse X chromosome (Fig. 1b) suggests that the E87-25 and CCR1 sequences may be close to the Phosphorylkinase (*Phk*) locus¹⁵ and distal to the *Bpa* locus¹⁶. The gene mutated in *Bpa* may be analogous to the locus controlling the human congenital disease chondrodysplasia punctata (*CDPX*), localized in pter-p22.32 on the basis of a number of phenotypic similarities¹⁷.

According to our results, the short arm of the human X

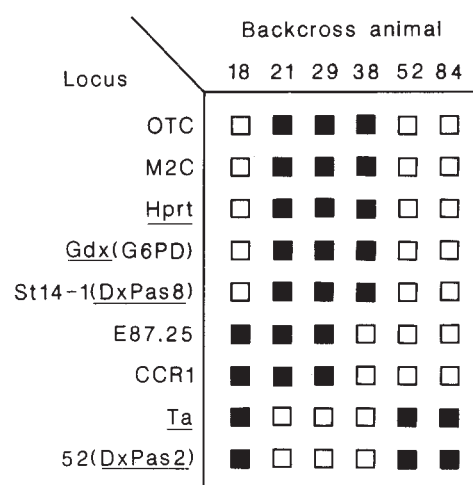


Fig. 3 Use of pedigree analysis of selected progeny from the *Mus spretus* backcross to localize the CCR1 and E87-25 defined loci between the *St14-1* defined (*DXPas8*) locus and the *Ta* coat colour gene. The haplotypes illustrated for female progeny correspond to that of the X chromosome of maternal origin. ■, B6CBAR1-derived allelic form of the locus, □, SPE/Pas-derived form of the locus. Numbers identify different animals from the backcross progeny. (For details of the cross and mouse strains used see refs 11-14).

chromosome would be represented in the mouse by at least three blocks of genetic material. The first, equivalent to the material between the centromere and *Otc* (Xp21) in man extends from the centromere to a position proximal to the *Hprt* locus and includes *A-raf* (ref. 12), *M2C* (*DXPas7*) (in preparation) and synapsin (*Syn-1*) loci¹⁸. The second block, equivalent to material between Xp21 and Xp22-3, is represented by the region on the mouse chromosome including the DMD/Becker muscular dystrophy (BMD) homologue and possibly *Bpa*. The third region, constituted by the telomeric region of the mouse X, would contain the steroid sulphatase locus (*Sts*), in principle homologous to the same locus on the tip of the human short arm (Xp22-3), and the closely linked *Hyp* and *Gy* genes, one of which is the probable equivalent to the HPDR locus in man (in Xp22)¹⁹⁻²².

On the basis of data suggesting that the *mdx* mutation lies within or close to the region bounded by the *G6pd* (*G6PD*) and *DXPas8* (*St14-1*) loci, we have previously suggested that this locus may well be equivalent to Emery Dreifuss muscular dystrophy (EMD)¹⁴ which shows tight linkage to the coagulation FVIII gene and to the *St14* (*DXS52*) locus in man^{6,7}. This is to some extent confirmed by differences in the histology and pathology of both DMD and BMD in man compared to that of the *mdx* mutation (J. Harris and G. Butler-Browne, personal communication). Such differences in pathology between man and mouse cannot however provide real evidence as to whether it is the same gene or not which is mutated in the two species. For instance mutations in the *Hprt* gene are known to cause Lesch Nyhan's disease in human males whilst mutations in the *Hprt* gene in male mice may well be asymptomatic²³.

The DMD complex in the mouse maps close to the region homologous to Xq26-Xqter in man which contains the gene responsible for Emery Dreifuss myopathy and suggests that at least two genes implicated in muscle function, the EMD and DMD equivalents, are relatively near each other on the mouse X chromosome. Our positioning of the DMD probes close to the *Phk* locus implies that there is another skeletal muscle gene in this relatively small chromosomal region. As the *Phk* mutation is without deleterious effects in the mouse, it must remain a possibility that unrecognized deficiencies in phosphorylase kinase are present in some DMD patients with large deletions (as is the case for some glycerokinase deficiencies)²⁴.

The proximity of the *mdx* mutation to both the putative EMD and the DMD loci makes it difficult to draw definite conclusions as to the disease to which *mdx* is homologous. Whereas we feel that the published genetic distances for *Hprt-mdx*, *Hq-mdx*, and *Ta-mdx* (refs 25 and 26) and (Peters *et al.* in preparation) make it more likely that *mdx* corresponds to EMD, both the proximity of the DMD locus and its complexity may mean that a resolution by classical genetic means will require study of the St14-1 probe and probes spanning the entire DMD locus in man in a cross segregating for the *mdx* mutation.

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1. Ray, P. N. *et al.* *Nature* **318**, 672-675 (1985).
2. Monaco, A. P. *et al.* *Nature* **323**, 646-650 (1986).
3. Monaco, A. P. *et al.* *Nature* **316**, 842-845 (1985).
4. Monaco, A. P. & Kunkel, L. M. *Trends Genet.* **3**, 33-37 (1987).
5. Bulfield, G., Siller, W. G., Wright, P. A. L. & Moore, K. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1189-1192 (1984).
6. Yates, J. R. W. *et al.* *J. med. Genet.* **29**, 587-590 (1986).
7. Thomas, N. S. T. *et al.* *J. med. Genet.* **23**, 596-598 (1986).
8. Worton, R. G., Duff, C., Sylvester, J. E., Schmickel, R. D. & Willard, H. F. *Science* **224**, 1447-1448 (1984).
9. Avner, P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).
10. Russell, L. B. in *Cytogenetics of the Mammalian X Chromosome* (ed. Sandberg, A.) 205-250 (Liss, New York, 1983).
11. Amar, L. C., Arnaud, D., Cambrou, J., Guénet, J. L. & Avner, P. R. *EMBO J.* **4**, 3695-3700 (1985).
12. Avner, P., Bucan, M., Arnaud, D., Lehrach, H. & Rapp, U. *Somatic Cell molec. Genet.* **13**, 267-272 (1987).
13. Dautigny, A. *et al.* *Nature* **321**, 867-869 (1986).
14. Avner, P., Amar, L., Arnaud, D., Hanauer, A. & Cambrou, J. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1628-1633 (1987).
15. Huijings, F., Eicher, E. M. & Coleman, D. L. *Biochem. Genet.* **193**-196 (1973).
16. Phillips, R. J. S., Hawker, S. G. & Moseley, H. J. *Genet. Res.* **22**, 91-99 (1973).
17. Happle, R., Phillips, R. J. S., Roessner, A. & Junemann, G. *Hum. Genet.* **63**, 24-27 (1983).
18. Yang-Feng, T. L., de Gennaro, L. J. & Francke, U. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8679-8683 (1986).
19. Eicher, E. M., Southard, J. L., Sriver, C. R. & Glorieux, F. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 4667-4671 (1976).
20. Lyon, M. F. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **83**, 4899-4903 (1986).
21. Read, A. P. *et al.* *Hum. Genet.* **73**, 267-270 (1986).
22. Mächler, M. *et al.* *Hum. Genet.* **73**, 271-275 (1986).
23. Kuehn, M. R., Bradley, A., Robertson, E. J. & Evans, M. J. *Nature* **326**, 295-298 (1987).
24. Clarke, A. *et al.* *J. med. Genet.* **23**, 501-508 (1986).
25. Chapman, V. M., Murawski, M., Miller, D. & Swiatek, D. *Mouse News Lett.* **72**, 120 (1985).
26. Bulfield, G. & Isaacson, J. H. *Mouse News Lett.* **72**, 97 (1985).

A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases

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Using DNA transfection analysis¹ on NIH 3T3 cells², activated human oncogenes have been isolated from a variety of fresh solid tumours³. Thyroid neoplasias show a wide range of lesions varying from slowly progressive well-differentiated tumours to anaplastic highly malignant neoplasms⁴. Therefore they represent an attractive model to investigate the role of oncogene activation in different stages of the neoplastic state. Here we report the detection of transforming activity in DNAs extracted from five thyroid papillary carcinomas and two of their respective lymph-nodal metastases.

The analysis of the *Alu* pattern of 'foci' induced by these DNAs has led us to conclude that all these transforming activities

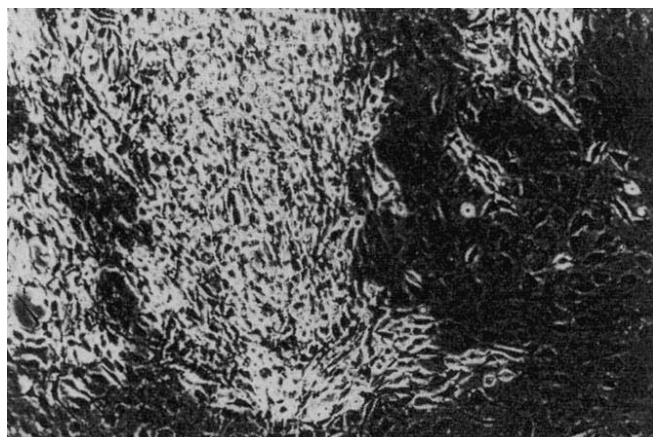


Fig. 1 Morphology of a transformed focus induced in NIH 3T3 cells by transfection of high M_r DNA from thyroid papillary carcinoma of patient 1. Magnification $\times 50$.

are mediated by a common oncogene. Moreover, the *Alu* pattern and the hybridization data obtained with tertiary transfectants indicate that this transforming gene is different from any other known viral and human oncogene. DNAs of high relative molecular-mass (M_r) obtained from 20 human thyroid papillary carcinomas have been assayed for their ability to induce focus formation on NIH 3T3 fibroblasts by the calcium phosphate DNA precipitation technique. In four of these patients it has been possible to analyse the transforming activity of DNAs extracted from their respective lymph-nodal metastases. DNAs extracted from five carcinomas (T28, T32, T22, T36 and T18) and two lymph-nodal metastases, M28 and M32 (obtained from the patients bearing carcinomas T28 and T32), induced foci of transformed cells. No lymph-nodal metastases were available from patients who donated samples T18, T36 and T22. The transforming activity of all the positive samples was remarkably high even in the first cycle (Table 1). Transformed NIH/3T3 cells had a very distinct morphological phenotype (Fig. 1). The cells were spindle-shaped or round, were very refractile and grew in a disordered way.

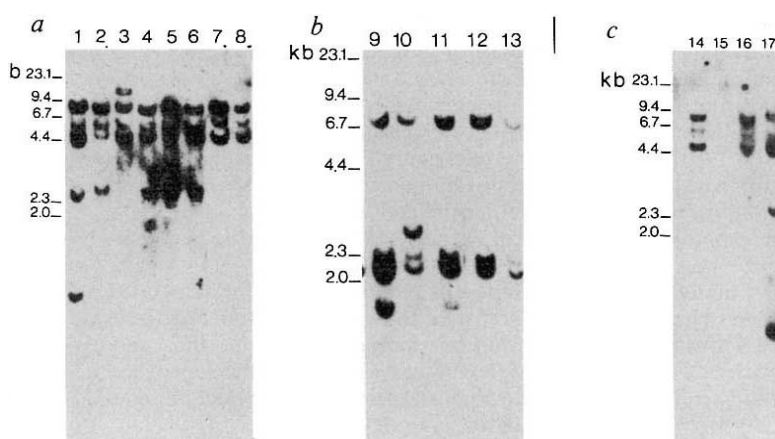
The morphology of the transformed cells, induced by the different DNA preparations, appeared to be a genetically stable property, and clonally derived cell lines retained the same morphology of the original focus produced by transformation.

DNAs of primary transfectants were shown to contain human repetitive sequences⁵ indicating that the transfectants had integrated exogenous human DNA (data not shown).

Transmissibility of transformed phenotype was retained in subsequent cycles of transfection with concomitant increased efficiency; all the cell lines established from first-, second- and third-cycle transfectants were able to grow into adult NIH OLAC mice, forming a palpable tumour in about 14 days (Table 1).

Second- and third-cycle transfectants were analysed by Southern blotting analysis and hybridization with Blur-8 probe⁵, specific for human repetitive sequences. All the foci were positive for human repetitive sequences. The pattern of *Alu*-positive bands, following digestion with different restriction enzymes, indicated the retention of the same specific human fragments in all the transfectants. In particular, after digestion with *Eco*RI, five bands of 0.8, 2.6, 5, 6.3 and 8.4 kilobases (kb) were constantly retained in secondary and tertiary transfectants from either T28 and M28 or T32 and M32 (Fig. 2a). In addition digesting with *Pst*I, four bands of 6.6, 2.3, 2.1 and 1 kb were constantly retained (Fig. 2b). The same results were obtained with the tertiary transfectants from T18, T36 and T22 (Fig. 2c), thus indicating that the same oncogene is activated in these five papillary carcinomas and in two of their respective metastases.

Fig. 2 Detection of human sequences in NIH 3T3 cells transformed by DNA of two thyroid papillary carcinomas and their respective lymph-nodal metastases. High M_r DNA (25 μ g) was digested with *a*, *c*, *EcoRI* *b*, *PstI*, electrophoresed, blotted and hybridized with a 32 P-labelled nick-translated 0.3-kilobase (kb) cloned repetitive sequence DNA fragment purified from plasmid BLUR-8 (2×10^6 c.p.m. ml^{-1}). Lanes 1, 2, 9 and 13, cellular DNAs isolated from two representative third-cycle transfectants of T28; lanes 3, 4 and 10, cellular DNAs isolated from two representative third-cycle transfectants of M28; lanes 5, 6 and 11, cellular DNAs isolated from two representative third-cycle transfectants of T32; lanes 7, 8 and 12, cellular DNAs from two representative third-cycle transfectants of M32; lanes 14, 16, 17, cellular DNAs from third-cycle transfectants of T22, T18 and T36, respectively; lane 15, cellular DNA from untransfected NIH 3T3 cells. Size markers, *HindIII* fragments of λ DNA.



To test the possibility that the detected oncogene was a member of the *ras* gene family, we analysed transfected DNAs by Southern blotting for the presence of human related sequences. None of the *ras* gene probes detected sequences other than those constitutively present in mouse (data not shown). Moreover our transforming gene lacks detectable homology to *sis*, *fos*, *fes/fps*, *fms*, *N-myc*, *raf*, *fgr*, *myb*, *src*, *myb*, *B-lym*, *p53*, *ets*, *erb-B* or *rel*. Finally comparison of *Alu*-positive fragments from second- and third-cycle transfectants with human specific fragments of *met*⁶, *mcf-2*⁷, *met*⁸, *trk*⁹, *ret*¹⁰, *neu*¹¹ and *dbl*¹² oncogenes indicates a lack of homology between our transforming gene and any of these recently detected oncogenes, suggesting that we have identified a new human transforming gene.

To clone the transforming gene of the papillary thyroid cancer we have constructed a phage library by inserting into a λ vector size-fractionated *EcoRI* partial digests of DNA from a tertiary transfectant derived from T28. From the library containing 5×10^5 phage clones, we obtained three overlapping phages that possessed human repeated DNA sequences (Fig. 3). These clones contain the five *EcoRI* *Alu*-positive fragments detected in all the transfectants.

We presume that the transforming gene was somatically activated in the tumour cells, as we have not observed a transforming activity in human DNA extracted from normal thyroid and lymphoid tissues obtained from the same patients (Table 1). Moreover, the remarkable high transforming activity of the human tumour DNAs, directly prepared from fresh surgical

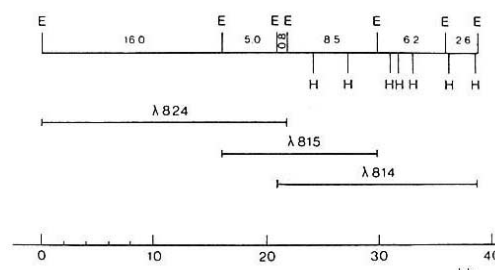


Fig. 3 A composite partial restriction endonuclease map of human transforming sequence from thyroid papillary carcinomas. Genomic DNA of a third-cycle transfectant from T28 was partially digested with *EcoRI* to select for fragments of 10–20 kb and cloned in the EMBL4 λ cloning vector. The library was screened with 32 P-labelled nick-translated BLUR-8 DNA as a probe. Three overlapping representative phage clones obtained are shown below the map. Sizes of restriction fragments shown in kb. Restriction endonuclease sites: E, *EcoRI*; H, *HindIII*.

specimens, as well as the presence of the same oncogene in five primary tumours and two metastases of different patients, make it unlikely that this new oncogene could be activated following the transfection process.

Because papillary carcinomas have been identified as the prevalent histotype of thyroid cancer following accidental or therapeutic irradiation¹³, it is interesting that the patient who

Table 1 Transformation of NIH 3T3 cells with DNAs from thyroid neoplasias

Code no.	Source of DNA	Primary foci	Tumour induction	Secondary foci	Tertiary foci
T32	Tumour of patient 1	8/4	4/4*	200/4	180/4
M32	Lymph-nodal metastases of patient 1	10/4	4/4	160/4	167/4
N32	Normal thyroid of patient 1	0/4	—	—	—
L32	Lymphocytes of patient 1	0/4	—	—	—
T28	Tumour of patient 2	80/4	4/4	148/4	159/4
M28	Lymph-nodal metastases of patient 2	20/4	4/4	160/4	166/4
L28	Lymphocytes of patient 2	0/4	—	—	—
T18	Tumour of patient 3	4/4	4/4	93/4	101/4
N18	Normal thyroid of patient 3	0/4	—	—	—
T22	Tumour of patient 4	6/4	4/4	68/2	73/2
L22	Lymphocytes of patient 4	0/4	—	—	—
T36	Tumour of patient 5	10/4	4/4	50/2	64/4
—	Tumours of patients 6–20	0/44	—	—	—
—	Lymph-nodal metastases of patients 6 and 7	0/8	—	—	—

Results are shown as (number of foci)/(total dishes examined).

NIH/3T3 (1.5×10^5) cells were transfected with 80 μ g of high-molecular-weight DNA by the calcium phosphate method¹.

* NIH/OLAC adult mice were injected subcutaneously with 1×10^6 cells of each cell line. Tumour formation was monitored for up to 30 days. Tumours appeared in two weeks.

donated samples T28 and M28 is reported to have been accidentally exposed to a high dose of ionizing radiations seven years before the occurrence of the thyroid neoplasia. These data also indicate that the same oncogene is activated in five thyroid papillary carcinomas, strongly suggesting tissue-specific activation for this putative new oncogene. Further investigations on different histological types of human thyroid carcinomas are in progress to ascertain whether the activation of this oncogene is a common feature of thyroid cancer or is related to the histological type.

Finally, this is the first report of a transforming gene associated with thyroid cancer and our data show that the same oncogene is activated in primary tumour and metastases of the same patient. This is also one of the few cases in which a new oncogene has been isolated from specimens obtained from several patients carrying the same histological type of tumour.

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1. Graham, F. L. & van der Eb, A. J. *J. Virol.* **52**, 456-467 (1973).
2. Iainchill, J. L., Aaronson, S. A. & Todaro, G. S. *J. Virol.* **4**, 549-553 (1960).
3. Pulciani, S. *et al. Nature* **300**, 539-542 (1982).
4. Heitz, P., Moser, H., Staub, J. J. *Cancer* **37**, 2329-2337 (1976).
5. Jelinek, W. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1398-1402 (1980).
6. Cooper, C. S. *et al. Nature* **311**, 671-673 (1983).
7. Fasano, O., Birnbaum, D., Edlund, L., Fogh, J. & Wigler, M. *Molec. cell. Biol.* **4**, 1695-1705 (1984).
8. Padua, R. A., Barrass, N. & Currie, G. A. *Nature* **311**, 671-673 (1984).
9. Martin-Zanca, D., Hughes, S. H. & Barbacid, M. *Nature* **319**, 743-748 (1986).
10. Takahashi, M., Ritz, J. & Cooper, G. M. *Cell* **42**, 581-588 (1985).
11. Schechter, A. L. *et al. Nature* **312**, 513-516 (1984).
12. Eva, A. & Aaronson, S. A. *Nature* **316**, 273-275 (1985).
13. Turrin, A., Pilotti, S. & Basso-Ricci, S. *Int. J. Radiat. Oncol. Biol. Phys.* **11**, 2149-2154 (1985).

The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants

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Crown gall and hairy root formation in plants involve the transfer of a DNA segment (T-DNA) from *Agrobacterium* to plant cells. The T-DNA, along with the *vir* genes which are essential for the DNA transfer process, is carried on a large tumour-inducing (Ti) plasmid in *Agrobacterium tumefaciens* (see refs 1 and 2 for review). The transfer of T-DNA, the only known case of naturally occurring DNA transfer from bacteria to plants, has been extensively exploited for the genetic transformation of plants³. Conjugal transfer of DNA between bacteria involves the transfer (*tra*) and mobilization (*mob*) gene products. The *mob* proteins nick DNA at the origin of transfer (*oriT*) and initiate single-stranded transfer of the DNA into the recipient bacterium (see ref. 4 for review). We report here that the mobilization functions on the naturally occurring wide-host-range plasmid RSF1010 can mediate the transfer of plasmids from *Agrobacterium* into plant cells as well as transfer between bacteria. This suggests that plants have access to the gene pool of Gram-negative bacteria.

The T-DNA region is flanked by two imperfect direct repeats of 25 base pairs (bp), called the left and right borders, which are important in the transfer process and define the region of DNA to be transferred⁵⁻⁷. To investigate the function of these 25 bp sequences in plant transformation, we constructed a series

Table 1 Plant transformation by various plasmids

Plasmids in LBA4404	Relevant characteristics	Plant transformation
pVW170	see Fig. 2A	None
pVW229	<i>oriT</i> on 153-bp fragment	None
pVW243	<i>oriT</i> on 1.4-kb fragment	None
pVW211	4.0-kb fragment of pSUP104	None
pJB125	<i>Tc^r</i> gene Δ of pSUP104	None
pVW170 + pJB125		None
pVW229 + pJB125		High
pVW243 + pJB125		High
pVW211 + pJB125		Low

The plasmids were transferred into LBA4404 and tested in plant transformation experiments, and transformation frequencies calculated, as in Fig. 1. 'None' means that no transformants were obtained when 10^9 bacteria ml^{-1} were used in the experiment, 'low' means that 1-2 transformed calli per leaf piece were obtained when 10^9 bacteria were used, and 'high' means that 1-2 calli per leaf piece were obtained when 10^6 bacteria ml^{-1} were used.

of plasmids based on the bacterial vector pSUP104 (ref. 8). These plasmids (Fig. 1) were mobilized into the disarmed *A. tumefaciens* strain LBA4404 (ref. 9), which contains the *vir* genes but not the T-DNA, to test them in plant transformation. Plant transformation still occurs in a binary system of this sort, in which the T-DNA and the *vir* genes are on separate replicons in *Agrobacterium*^{9,10}. All plasmids gave transformed plants, including pJP181, the plasmid carrying no T-DNA border sequences, and all were found to transform at approximately the same frequency. This showed that in this case the different border configurations had no effect on the transformation frequency and, because the plasmid containing no border sequences also transformed at high frequency, that there was no requirement for any border sequence. Therefore either the 25-bp sequence is not required for transformation by the binary system, or there is some other sequence or function on these plasmids that replaces the requirement for a border sequence.

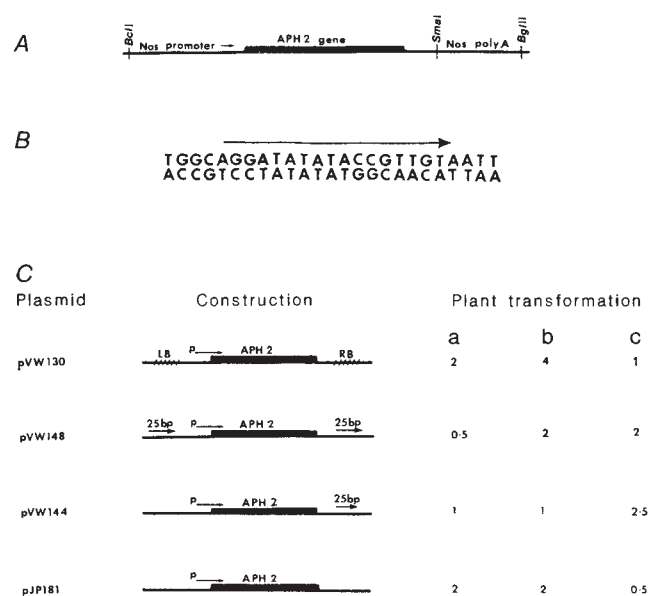
The implication that a border sequence is not required for transformation is contradicted by other reports^{11,12}. Both of these groups used a deletion derivative of the wide-host-range plasmid RK2 (ref. 13) rather than pSUP104 as the replicon for their binary vectors. To confirm these results the plant selectable marker from pJP181 with no border sequences and from pVW144 with a single border sequence were cloned into the plasmid pRK290 (ref. 14), a deletion derivative of RK2, to give plasmids pVW170 and pVW171 respectively (Fig. 2A). These plasmids were transferred into LBA4404 and tested in plant transformation.

Although pVW171 was capable of transforming plants, pVW170 was not. This result confirms that a border sequence is necessary when pRK290 is the replicon and shows that the 'border' function is essential in the binary transformation system. Therefore the vector pSUP104 must have a sequence or function that replaces the requirement for a border sequence. Because the frequency of transformation is no higher when borders are present the 'border' function on pSUP104 is at least as efficient as the natural borders.

The plasmid pSUP104 was made by joining the *E. coli* vector pACYC184 (ref. 15) to a portion of the wide-host-range plasmid RSF1010 (ref. 16) and selecting for a plasmid that could be mobilized to, and stably maintained in, a Gram-negative organism other than *E. coli*⁸. The resulting plasmid pSUP104 has the replicon and resistance genes for the antibiotics tetracycline and chloramphenicol from pACYC184 and the replication and mobilization functions of RSF1010. To identify the region of pSUP104 with the border function, several parts of the plasmid were cloned into the *EcoRI* site of pVW170 (Fig. 2B). The resulting clones were transferred to LBA4404 and tested in plant

Fig. 1 Construction of vectors to determine the function of borders in plant transformation. **A**, The structure of the plant selectable marker which is the same in all plasmids. The aminoglycoside phosphotransferase II gene from Tn5 (ref. 23) is fused at its 3' end to a 250-bp *Sau3A* fragment carrying the polyadenylation site from the nopaline synthase gene²⁴. This was ligated using *Sma*I linkers into a *Sma*I site 170-bp after the termination codon for the APHII gene creating a *Bgl*III site at the 3' end. The promoter for the nopaline synthase gene²⁴ is fused to the 5' end of the APHII gene to allow its expression in plant cells. The fusion junction is 2 bp upstream from the start codon of the APHII gene and 28 bp downstream of the start of transcription of the nopaline synthase promoter. The entire selectable marker can be transferred on a *Bcl*I-*Bgl*III fragment. **B**, The synthetic 25-bp sequence, used in some of the constructions, is the 25-bp sequence at the right border of the octopine TL DNA from the Ti plasmid pTi15955 (ref. 5). Arrow, orientation of this sequence as shown in the plasmid diagrams. **C**, The arrangement of the border sequence around the selectable marker is shown for the four plasmids. Plasmid pVW130 has border regions from the TR DNA of the Ti plasmid pTiB6, the right border is cloned on a 3-kb *Eco*RI fragment and the left border is cloned on a 1-kb *Xho*I-*Eco*RI fragment^{5,25,26}. Plasmid pVW148 has a synthetic border sequence on each side of the selectable marker in the correct orientations for the left and right borders. Plasmid pVW144 has a single 25-bp sequence in an orientation equivalent to having a single right border and pJP181 has no border sequences. Transformation efficiency of each plasmid is also shown as number of kanamycin resistant calli per leaf piece at 10^5 bacteria per ml in three separate experiments (a, b and c).

Methods. All selectable marker and border constructions were cloned into the unique *Bam*HI site on pSUP104. The plasmids were mobilized into *A. tumefaciens* strain LBA4404 by triparental crosses using pRK2013 as a helper plasmid. Plant transformation experiments were carried out by the explant method²⁷ as follows: sterile *Nicotiana plumbaginifolia* leaf pieces were dipped in an overnight culture of the *Agrobacterium* strain, which had been spun down and resuspended in 0.85% saline, blotted on filter paper and placed on a feeder layer of *Glycine max* suspension culture cells on MS4 medium (MS agar²⁸ containing 0.3 mg l⁻¹ indole acetic acid and 10 mg l⁻¹ dimethylaminopurine). After three days the leaf pieces were transferred to MS4 plates containing carbenicillin at 200 mg l⁻¹ and kanamycin at 50 mg l⁻¹. After three or four weeks transformed shoots were visible on the leaf pieces. To try to distinguish any differences in transformation efficiency between vectors a system for measuring transformation frequency was developed (manuscript in preparation), which involved making serial dilutions of each bacterial culture, treating 12–15 randomly selected leaf pieces with each dilution, and scoring for the dilution that gave 1–2 kanamycin resistant calli per leaf piece. By this means differences in efficiency of transformation by different plasmids can be determined. Transformed plants obtained from the four different vectors were analysed. Results from Southern blotting of the plant DNA and hybridization to labelled probes for the plasmids, including one for the APHII gene, were consistent with one or two copies of each plasmid being integrated into the plant chromosomal DNA. Seed from transformed plants segregated in most cases at a normal mendelian ratio of three resistant seed to one sensitive which is consistent with the inheritance of a single, dominant nuclear marker^{29–31}.



transformation. The only plasmids capable of transforming plants, pVW210 and pVW214 (Fig. 2), have the same 2.9-kilobase insert but in different orientations. Therefore the 'border' function carried by pSUP104 must be on this 2.9 kb *Ava*I fragment, which is entirely from RSF1010. There was a possibility that this fragment could replace the function of a border sequence by possessing a sequence similar enough to the border sequence to have equivalent activity. Analysis of the DNA sequence of the 2.9-kb *Ava*I fragment (not shown) showed that there was no significant similarity to the 25-bp T-DNA border sequence, implying that there is some function on this DNA fragment that eliminates the need for a border sequence.

Previously published data on RSF1010 (refs 17, 18) or R1162 (refs 19, 20) (a plasmid very similar to RSF1010) have mapped functions such as the *rep* genes, *oriT* and *oriV* (Fig. 2). The 2.9-kb *Ava*I fragment contains the sequences for *oriV*, the vegetative origin of replication; *oriT*, the origin of transfer of the DNA; a part of the *repB* gene and the gene or genes that specify the *mob* region. The most likely candidate for the border function is the *oriT* sequence, acting with or without the *mob* genes. The 2.9-kb fragment was cloned into pUC8 (ref. 21), a small non-mobilizable *E. coli* plasmid, and the resulting plasmid pVW226 was mobilizable at high frequency between *E. coli* strains with pRK2013, which contains the *tra* genes of RK2 (ref. 14), as a helper plasmid. This implied that the RSF1010 *oriT* and entire *mob* region are on this fragment, as *oriT* sequences are usually very specific to their compatible *mob* genes⁴. The plasmid pRK290 is a deletion derivative of the self-transmissible plasmid RK2 and although it still contains *oriT* it may not carry the entire *mob* gene region as compatible *mob* genes as well as *tra* genes are provided in *trans* by the helper plasmid pRK2013

which is used to mobilize pRK290. This could explain why pRK290 lacking a border sequence is not transferred to plant cells.

To determine whether the *oriT* sequence and/or the *mob* genes were involved in the transfer of DNA to plants by pSUP104 a series of experiments was designed. First, the *oriT* region was cloned separately, as a 153-bp *Taq*I fragment⁴ in pUC8 to give plasmid pVW228, which was mobilized between *E. coli* strains at a frequency of 10^{-4} per recipient only when pRK2013 and pSUP104 together provided *tra* and *mob* functions respectively in *trans*. A larger fragment of DNA (1.4-kb fragment from the *Ava*I site to a *Cfr*101 site) which carried the *oriT* DNA and an extra repeated sequence which may be part of the *oriT* recognition site²⁰ was cloned into pUC8 to give pVW235 and this plasmid was mobilized at a tenfold higher frequency between *E. coli* strains. These two fragments carrying the *oriT* sequences were cloned into the *Eco*RI site of pVW170 to give pVW229 and pVW243 respectively, transferred to LBA4404 and tested in plant transformation. In neither case was transformation obtained. Therefore the *oriT* sequence alone is not sufficient for the transfer of DNA into the plant.

If the *oriT* sequence and *mob* genes act together to transfer the DNA into plants it should be possible to provide the *mob* genes in *trans* to mobilize the plasmid carrying the *oriT* sequence. This was tested using pJB125, a derivative of pSUP104 that has a deletion in the tetracycline gene (J. Beynon, unpublished data). Deletion of this gene made it possible to select for two plasmids in the same strain. This plasmid was mobilized into LBA4404 together with derivatives of pVW170 (Table 1). Both plasmids carrying *oriT* sequences were complemented in *trans* by pJB125 and high-frequency plant transformation was

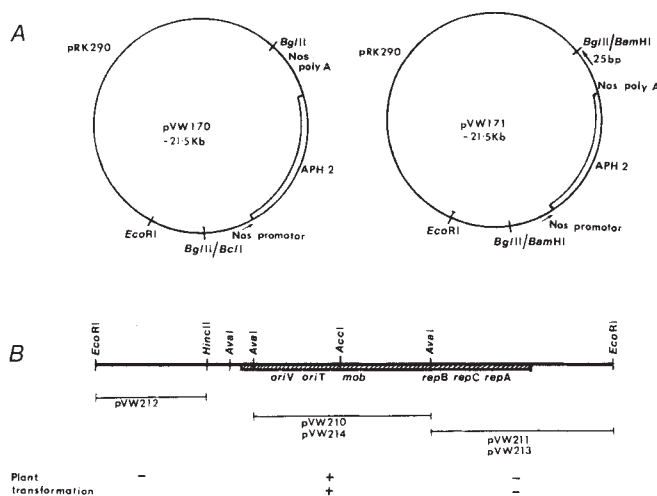


Fig. 2 **A**, The *BclII*-*BglIII* fragment carrying the plant selectable marker (Fig. 1) was cloned into the *BglIII* site of pRK290 to give pVW170. The *BamHI* fragment from pVW144 carrying the selectable marker and a 25-bp border sequence was cloned into the *BglIII* site of pRK290 to give pVW171. These two plasmids are identical except that pVW171 has a 25-bp border sequence next to the selectable marker. **B**, Subcloning regions of pSUP104. The fragments of pSUP104 shown in the figure were isolated, *EcoRI* linkers were added and they were cloned into the *EcoRI* site of pVW170. The plasmids obtained were mobilized into LBA4404 and tested in plant transformation as in Fig. 1. Two of the plasmids, pVW210 and pVW214, were positive for transformation whereas the other three were negative. Shaded line, the part of pSUP104 contributed by RSF1010. The positions of *oriV*, *oriT* and the *mob* and *rep* genes are from previously published information¹⁷⁻²⁰.

obtained. To show that this was not due to recombination in *Agrobacterium* between the homologous regions of the two plasmids (pJB125 also carries *oriT*) complementation of pVW211 was tested. This plasmid carries a 4.0-kb *EcoRI*-*AvaI* fragment of pSUP104 that has no plant transformation capability (Fig. 2) and was mobilized to plant cells at low frequency when pJB125 was provided in *trans*. The transfer is presumably due to recombination between the two plasmids and as this occurs at low frequency and pVW211 has a 4.0-kb region of similarity to pJB125 compared to about 153-bp similarity for pVW229, which is transferred at high frequency, this indicates that the complementation of pVW229 and pVW243 by pJB125 is not due to recombination. However, the unlikely possibility of a highly efficient site-specific recombination around the *oriT* site has not been ruled out. That pVW229 but not pVW170 is complemented by pJB125 shows that the *oriT* sequence is essential for the transfer of DNA to plants.

An *in vitro* transcription-translation assay carried out on the 2.9-kb *AvaI* fragment cloned in pVW226 showed that at least three proteins of relative molecular mass (M_r) 72,000, 14,700 and 13,500 are synthesized from this DNA. These proteins may all be involved with the *mob* function. Three proteins of M_r 60,000 (60K), 16K and 11K have been implicated in mobilization of *ColEI* (ref. 4) and it has been suggested that four *trans*-active products could be involved in mobilization of the plasmid R1162 (ref. 20). Therefore it is expected that RSF1010 may have three or four *mob* proteins. To identify the 72K protein as a *mob* protein and to determine if it was required for plant transformation, a frame shift mutation was made at the *AccI* site in the 72K protein gene. The DNA was cut with *AccI*, the sticky ends filled in and the DNA religated to introduce an extra two base pairs into the DNA and alter the reading frame of the 72K protein. This resulted in complete removal of this protein whereas the other two proteins were still made. The pUC8 clone containing this mutated fragment, pVW241, was mobilized between *E. coli* strains at a frequency of about 10^{-4} per recipient as opposed to a mobilization frequency of 10^{-2} per recipient for the unmutated plasmid pVW226. This shows that the 72K protein is involved in, but is not essential for, mobilization between *E. coli* strains. Presumably its function is replaced by a gene encoded either by the host bacterium or by the helper plasmid pRK213. The *EcoRI* fragment from pVW241 carrying the 2.9-kb DNA fragment with the mutated *mob* gene was cloned into the *EcoRI* site of pVW170 to give pVW244. When this plasmid was transferred into LBA4404 and tested in plant transformation experiments as described, it was incapable of transforming plants. In this experiment a reduction of transformation frequency equivalent to the reduction in transfer frequency between *E. coli* strains would have been detectable because

plant transformation with LBA4404 carrying the unmutated plasmid (pVW210) can be detected with bacterial densities as low as 10^5 ml⁻¹. Therefore this 72K *mob* protein is essential for transfer of DNA into plants.

Our results show that the mobilization functions of a bacterial plasmid can promote the transfer of a plasmid carrying these functions into plant cells. Both the *oriT* site and at least some of the *mob* genes are essential for this transfer. The plasmid concerned, RSF1010, is a small, naturally occurring, wide-host-range plasmid that can be transferred amongst Gram-negative bacteria. Therefore this system has the potential to allow transfer of DNA from different bacteria into plant cells (Fig. 3). The evolutionary significance of this result lies in the possibility of gene flow from bacteria to plants which specifically means that any gene normally found in a Gram-negative bacterium could be transferred by a plasmid such as RSF1010 into plants. It is quite likely that the *mob* and *oriT* regions of other plasmids could function in the same way as those of RSF1010; presumably however in this system the *vir* genes from the Ti plasmid replace some functions normally carried out by the *tra* genes during

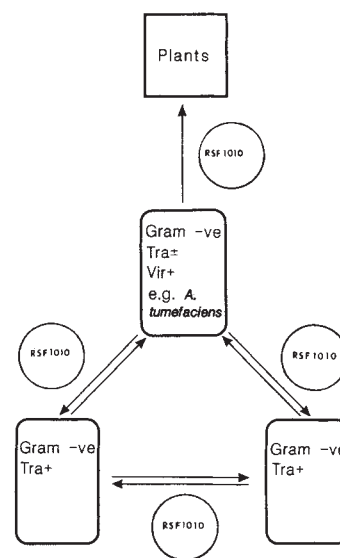


Fig. 3 Diagram to represent the potential spread of RSF1010 amongst plant and bacterial populations. The plasmid can be mobilized into *Vir*⁺ bacteria like *Agrobacterium tumefaciens* and thence into plant cells, or it can be transferred amongst other Gram-negative bacteria. At any stage it could incorporate bacterial DNA which could then be transferred to the next recipient.

bacterial transfer. Experiments with several different *vir* mutants have indicated that at least some of the *vir* genes are required for transfer of plasmids carrying *oriT* and *mob* instead of the 25-bp border sequence. Although *tra* genes do not need to be exactly compatible with *mob* genes and *oriT* sequences there is some specificity in their interaction⁴ and therefore not all *mob* and *oriT* functions may be compatible with the *vir* genes. This, and the possible involvement of other *tra* genes, remains to be investigated. Furthermore other bacteria that interact with plants may have *vir* genes capable of mobilizing bacterial plasmids into plant cells.

DNA transfer between bacteria occurs by replicative transfer of single strands⁴. Recent evidence indicates that an early step in T-DNA transfer, after induction of the *vir* genes, is the formation of single-stranded linear molecules corresponding to the bottom strand of the T-DNA on the Ti plasmid²². Because it has now been shown that components of the bacterial system can operate for transfer of DNA from *A. tumefaciens* to plants this gives strong support to the previous hypothesis that the T-DNA is transferred to plants as a single-stranded molecule²². Transfer of the T-DNA in a normal infection system results in integration into the plant DNA at the right and left border but with a higher degree of specificity at the right border^{6,7}. This may simply arise because the right border leads the way into the plant cell and may be protected by *vir* proteins bound to it, by analogy to the bacterial transfer system, where *mob* proteins are bound to the end of the single-stranded DNA molecule being transferred⁴. It is not known whether the 25-bp sequence is involved in any way in the integration process but it is obviously not essential. It remains to be shown whether the DNA transferred by the *oriT/mob* plasmids is integrated at the *oriT* site in the same way as the T-DNA is integrated at the right border.

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- Lichtenstein, C. *Nature* **322**, 682-683 (1986).
- Stachel, S. E. & Zambryski, P. C. *Cell* **47**, 155-157 (1986).
- Goodman, R. M., Hauptli, H., Crossway, A. & Knauf, V. C. *Science* **236**, 48-54 (1987).
- Willets, N. & Wilkins, B. *Microbiol. Rev.* **48**, 24-41 (1984).
- Barker, R., Idler, K., Thompson, D. & Kemp, J. *Plant molec. Biol.* **2**, 335-350 (1983).
- Zambryski, P., Depicker, A., Kruger, K. & Goodman, H. J. *Molec. appl. Genet.* **1**, 361-370 (1982).
- Holsters, M. *et al. Molec. gen. Genet.* **190**, 35-41 (1983).
- Simon, R., Priefer, U. & Puhler, A. in *Molecular Genetics of the Bacteria-Plant Interaction* (ed. Puhler, A.) 98-106 (Springer, Berlin, 1983).
- Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. *Nature* **303**, 179-180 (1983).
- de Fromand, A. J., Barton, K. A. & Chilton, M. *Bio/Technology* **1**, 262-269 (1983).
- Horsch, R. B. & Kle, H. J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4428-4432 (1986).
- Jen, G. C. & Chilton, M. J. *Bact.* **166**, 491-499 (1986).
- Burkhardt, H. J., Reiss, G. & Puhler, A. J. *gen. Microbiol.* **114**, 341-348 (1979).
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7347-7351 (1980).
- Chang, A. C. Y. & Cohen, S. N. *J. Bact.* **134**, 1141-1156 (1978).
- Guerry, P., Van Embden, J. & Falkow, S. *J. Bact.* **117**, 619-630 (1974).
- Haring, V. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 6090-6094 (1985).
- Persson, C. & Nordstrom, K. *Molec. gen. Genet.* **203**, 189-192 (1986).
- Meyer, R. J., Lin, L.-S., Kim, K. & Brasch, M. A. in *Plasmids in Bacteria* (ed. Helinski, D. R., Cohen, S. N., Clewell, D. B., Jackson, D. A. & Hollaender, A.) 173-188 (Plenum, New York, 1985).
- Brasch, M. A. & Meyer, R. J. *J. Bact.* **167**, 703-710 (1986).
- Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).
- Stachel, S. E., Timmerman, B. & Zambryski, P. *Nature* **322**, 706-712 (1986).
- Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B. & Schaller, H. *Gene* **19**, 327-336 (1982).
- Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. & Goodman, H. J. *Molec. appl. Genet.* **1**, 561-573 (1982).
- Thomasow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P. & Nester, E. W. *Cell* **19**, 729-739 (1980).
- Ooms, G. *et al. Cell* **30**, 589-597 (1982).
- Horsch, R. B. *et al. Science* **227**, 1229-1231 (1985).
- Murashige, T. & Skoog, F. *Physiol. Pl.* **15**, 473-497 (1962).
- DeBlock, M., Herrera-Estrella, L., Van Montagu, M., Schell, J. & Zambryski, P. *EMBO J.* **3**, 1681-1689 (1985).
- Potrykus, I., Paszkowski, J., Saul, M. W., Petruska, J. & Shillito, R. *Molec. gen. Genet.* **199**, 169-179 (1985).
- Hain, R. *et al. Molec. gen. Genet.* **199**, 161-168 (1985).

Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene

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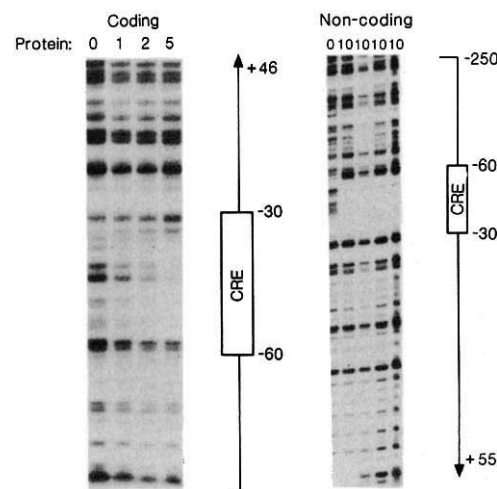
Many hormones act on neuroendocrine cells by activating second messenger pathways. Two of these, the phosphoinositol and cAMP-dependent pathways, cause changes in cellular activity through specific protein kinases. By phosphorylating cytoplasmic and nuclear proteins, these kinases apparently coordinate cellular processes, including the biosynthesis and release of neuropeptides. Somatostatin biosynthesis and release, for example, are both positively regulated by the second messenger cAMP in hypothalamic cells¹, and cAMP also induces somatostatin gene transcription 8-10-fold in transfected PC12 pheochromocytoma cells². Transcriptional induction requires a 30-nucleotide cAMP response element (CRE) which is conserved in other cAMP-responsive genes²⁻⁴. This element also confers cAMP responsiveness when placed upstream of the heterologous simian virus 40 (SV40) promoter. The somatostatin gene does not, however, respond to cAMP in mutant PC12 cells which lack cAMP-dependent protein kinase type II activity². Activation of somatostatin gene transcription may consequently require the phosphorylation of a nuclear protein which binds to the CRE. Using a DNase I protection assay, we have characterized a nuclear protein in PC12 cells which binds selectively to the CRE in the somatostatin gene. We have purified this protein which is of relative molecular mass 43,000 (*M*, 43K) by sequence-specific DNA affinity chromatography. This 43K CRE binding protein (CREB) is phosphorylated *in vitro* when it is incubated with the catalytic subunit of cAMP-dependent protein kinase. Stimulating PC12 cells with forskolin, an activator of adenylyl cyclase, causes a 3-4-fold increase in the phosphorylation of this protein. We conclude that the cAMP-dependent pathway may regulate gene transcription in response to hormonal stimulation by phosphorylating this CREB protein.

Somatostatin is a hypothalamic peptide that inhibits the release of several pituitary hormones, including growth hormone, prolactin, and thyrotropin⁵. Somatostatin release from hypothalamic neurons is, in turn, regulated by a number of classical neurotransmitters and neuromodulators which activate adenylyl cyclase⁶⁻⁸. Somatostatin gene transcription is also stimulated by agents which increase intracellular cAMP (ref. 2). The observation that transcriptional regulation by cAMP depends on a conserved promoter element has prompted us to ask whether a specific nuclear protein binds to this sequence.

We used a DNase I footprinting assay on nuclear extracts of PC12 cells to detect DNA-binding proteins which interact with the CRE. Increasing amounts of protein from the extract were incubated with a 5' end-labelled somatostatin promoter fragment. Samples were partially digested with DNase I and the products of digestion were resolved on 8% denaturing polyacrylamide gels. We observed a single footprint which covered 23 nucleotides on each strand of the DNA (Fig. 1). When run alongside DNA sequence markers (not shown), this footprint, extending from -55 to -32 relative to the transcription initiation site, mapped to the same region as the somatostatin CRE sequence. Approximately 10 µg protein from unfractionated extracts was required to see complete protection. Nuclear extracts prepared from either forskolin-treated or untreated PC12 cells contained the same amount of CRE-binding activity relative to total nuclear protein. To determine whether binding of this nuclear factor *in vitro* correlated with transcriptional activation by cAMP *in vivo*, we examined the somatostatin

Fig. 1 DNase I footprinting of somatostatin promoter fragments using unfractionated PC12 nuclear extract with (left) coding and (right) non-coding strand probes. Position of cAMP response element (CRE) compared with footprinted region is shown diagrammatically. Nucleotide positions relative to somatostatin transcription initiation point are listed alongside. Coding strand probe was prepared from plasmid $\Delta(-71)$ (ref. 2). Following digestion with *Hind*III, $\Delta(-71)$ plasmid was 5' end-labelled with T4 polynucleotide kinase and redigested with *Ava*II. The 140-bp *Hind*III-*Ava*II fragment was purified by polyacrylamide gel electrophoresis. Non-coding strand probe was isolated from plasmid $\Delta(-250)$ (ref. 2). DNA from this plasmid was digested with *Xba*I, 5' end-labelled, and redigested with *Sal*I. The 305-bp *Xba*I-*Sal*I fragment was also gel purified. Numbers above each lane, amount of unfractionated PC12 nuclear extract (in μ g) used for each footprint reaction. Protein concentration was determined according to Bradford¹⁵. After incubating nuclear extract with labelled DNA fragments, samples were treated with DNase I, and the products of digestion were resolved on 8% urea-polyacrylamide gels.

Methods. Nuclear extracts were prepared using a protocol adapted from Dignam *et al.*¹⁶. Cells (5×10^9) were harvested and spun at 2,000g for 10 min. The cell pellet was then resuspended in four packed cell volumes of buffer H (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF), 10 mM NaF, 1,000 U ml⁻¹ Trasylol (aprotinin)). PC12 cells were allowed to swell on ice for 15 min. Cells were lysed with 20 strokes of a Dounce homogenizer (B pestle) and nuclei were subsequently recovered by centrifugation at 3,000g for 8 min. The cloudy supernatant was decanted and the nuclear pellet was washed with an additional 25 ml buffer H. The nuclei were resuspended in four packed nuclear volumes of buffer D (50 mM Tris-HCl, pH 7.5, 10% sucrose, 0.42 M KCl, 5 mM MgCl₂, 0.1 mM EDTA, 20% glycerol, 2 mM DTT, 0.1 mM PMSF, 20 mM NaF, 1,000 U ml⁻¹ Trasylol) and were incubated on ice for 30 min with stirring. The viscous nuclear suspension was then centrifuged at 25,000g for one hour. The supernatant was collected, and the nuclear protein from this fraction was precipitated in 53% saturated NH₄SO₄. After centrifugation at 16,000g for 20 min (4 °C), the ammonium sulphate pellet was resuspended in 2–3 ml TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.1% Nonidet-P40, 1 mM PMSF, 0.1 M KCl) and dialysed for 3 h (4 °C) against 500 vol TM buffer. A stringy precipitate which formed during dialysis was removed by centrifuging the extract at 10,000g for 10 min. Typically, 20–30 mg nuclear protein were recovered per 5×10^9 PC12 cells. For footprint reactions, nuclear extract protein was incubated with 10 fmol ³²P-labelled DNA probe for 15 min on ice, 1 μ g poly (dI-dC) competitor DNA (no competitor DNA was used with purified fractions), in 2% polyvinyl alcohol, 25 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.05% Nonidet-P40, 0.1 M KCl. Samples were then warmed to 25 °C and diluted with an equal volume of 10 mM MgCl₂, 10 mM CaCl₂. Freshly diluted DNase I was added and reactions were allowed to proceed for 1 min at 25 °C. DNase digestion was terminated by the addition of 90 μ l 20 mM EDTA, 1% SDS, 0.2 M NaCl, 25 μ g transfer RNA. Samples were deproteinized by phenol-chloroform extraction and then ethanol precipitated.



promoter deletion mutant $\Delta(-48)$ CAT (ref. 2). We have shown elsewhere² that this fusion gene, extending to position -48 of the somatostatin promoter, is not transcriptionally responsive to cAMP. Likewise, we were unable to observe any protein binding to the mutant CRE sequence (data not shown).

To purify CREB, we constructed a sequence-specific DNA affinity column⁹. Affinity resin was prepared by self-ligating a double-stranded synthetic oligonucleotide containing a high-affinity CRE site to produce concatemers with an average of eight copies. The ligated DNA was attached covalently to cyanogen-bromide-activated CL-2B Sepharose beads. Unfractionated PC12 nuclear extracts were then passed over 1-ml affinity columns. After washing the columns repeatedly with low salt buffer (0.1 M KCl), the specific CRE binding protein was eluted with salt buffer containing 1.0 M KCl. Recovery of CREB protein, followed by footprint analysis, averaged 50% for each pass over the affinity column (Fig. 2a).

We monitored the purification of CREB protein by analysing the high salt eluates from successive column passes on SDS-polyacrylamide gels (Fig. 2b). Aliquots containing equivalent footprinting activity (100 footprint units; one unit is defined as the amount of CREB protein required to bind to the CRE sequence and protect it from digestion by DNase I) from each affinity pass were precipitated with trichloroacetic acid, acetone washed, and electrophoresed under denaturing conditions. We observed a band of relative molecular mass 43,000 (M_r 43K) which became the predominant protein in the extract after two passes over the affinity column (Fig. 2b). After four column passes, all protein bands were visibly reduced except for the 43K band.

To verify that the 43K protein which we had purified was CREB, we performed UV-cross-linking experiments (Fig. 3). Using this technique, individual DNA-binding proteins can be identified in crude nuclear extracts¹⁰. The DNA probe for cross-linking experiments was prepared by uniformly labelling a 160 base pair (bp) somatostatin promoter fragment with [α -³²P]dCTP and BUDR triphosphate. After incubating the DNA

probe with unfractionated nuclear extract, the mixture was exposed to ultraviolet light of wavelength 300 nm for one hour. Samples were digested with DNase I and micrococcal nuclease and then analysed on an SDS-polyacrylamide gel. We observed a single protein-DNA adduct of 43K which was resistant to further increases in DNase digestion (Fig. 3a). No DNase-resistant bands were observed when either extract protein or ultraviolet irradiation were omitted (data not shown).

We performed competition experiments to determine whether the 43K complex represented the specific binding of a nuclear protein to its target DNA sequence. When a 50-fold molar excess of unlabelled competitor DNA containing the CRE was added to the reactions, no cross-linked proteins were observed (Fig. 3a). By contrast, similar addition of competitor DNA containing the Sp1 binding site had no effect on the 43K complex (Fig. 3b). Because the migration of proteins does not appear to be significantly altered when cross-linked to small oligonucleotide fragments¹¹ we estimate that the actual M_r of the CRE-binding protein is near 43K.

The dependence of cAMP-regulated transcription on cAMP-dependent protein kinase activity² led us to examine the phosphorylation of CRE-binding protein by the catalytic subunit of cAMP-dependent protein kinase. Affinity purified extract was incubated with catalytic subunit of cAMP-dependent protein kinase and [γ -³²P]ATP. After electrophoresis, two major phosphoproteins of 30K and 43K were observed in addition to the autophosphorylated 41K catalytic subunit (Fig. 4). Affinity chromatography of this labelled extract showed that 43K phosphoprotein was eluted from the column only with high salt buffer. In contrast, the 41K catalytic subunit, although in large excess compared with CREB, was completely eluted from the column with low salt. Thus 43K protein retained high CRE binding affinity after phosphorylation with catalytic subunit.

To determine whether phosphorylation of CREB protein is stimulated by cAMP *in vivo*, we labelled PC12 cells overnight with ³²P-orthophosphate and then incubated these in the presence or absence of forskolin for one hour. Nuclear extracts were

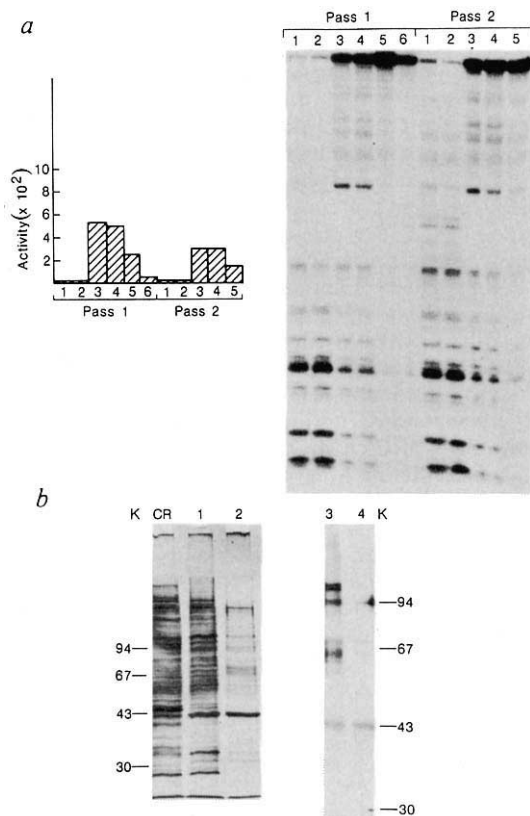


Fig. 2 Purification of CREB protein from PC12 nuclear extract by DNA affinity chromatography. Crude nuclear extract (25 mg protein) was preincubated with 125 μ g calf thymus DNA, 125 μ g poly(dI-dC) for 10 min on ice. The extract was passed over a 1-ml DNA affinity column equilibrated in buffer Z with 0.1 M KCl. Buffer Z contained 25 mM HEPES pH 7.8, 12.5 mM MgCl₂, 1 mM DTT, 20% glycerol, 0.1% Nonidet-P40. The column was washed with 6 ml buffer Z containing 0.1 M KCl. After collecting the wash fractions, the column was capped and 1 ml buffer Z containing 1.0 M KCl was added to the affinity resin. The resin was briefly stirred and then incubated for 20 min to allow dissociation of bound protein. The eluate was collected, an additional 1 ml of buffer Z with 1.0 M KCl was added to the resin and residual CRE-binding protein activity was collected. Additional passes over the affinity column using purified extracts were as described in Fig. 1 legend. **a**, CRE binding activity recovered from the column was monitored by DNase I footprinting assay using coding strand probe (Fig. 1). Right, elution profiles for two successive affinity column passes are shown. Lanes marked 1 denote flow-through fractions. Lanes marked 2 represent 0.1 M KCl wash fractions. Lanes marked 3, 4, 5, 6 represent successive 1-ml high salt (1.0 M KCl) eluate fractions. Bar graph on left, CRE-binding activity recovered from each corresponding fraction expressed in footprint units. **b**, SDS-polyacrylamide gel analysis of eluate fractions from DNA affinity column. Ticks alongside each gel, protein markers. Sizes are M_r in thousands. CR, crude (unfractionated) extract; lanes marked 1, 2, 3, 4 represent first, second, third and fourth pass high salt eluates, respectively. Third and fourth pass high salt fractions shown on right panel originated from a different nuclear extract preparation from those on left panel. Samples containing 100 footprint units each (except crude extract sample which contained 20 footprint units) were precipitated with 20% trichloroacetic acid plus 1 mM deoxycholate, washed with acetone and resuspended in sample buffer. Following electrophoresis over 8% SDS-polyacrylamide gels under reducing conditions, proteins were silver-stained according to Morissey¹⁷. Arrow, putative 43K CRE-binding protein. **Methods.** The sequence-specific DNA affinity column was constructed according to Kadonaga and Tjian⁹. A double-stranded oligonucleotide extending from -62 to -37 and containing *Bam*HI cohesive ends was synthesized. The oligomers were annealed, 5' phosphorylated, and ligated to produce concatemers with an average length of 300 bases. The DNA (400 μ g) was covalently attached to 10 ml of CnBr-activated CL-2B Sepharose beads⁹.

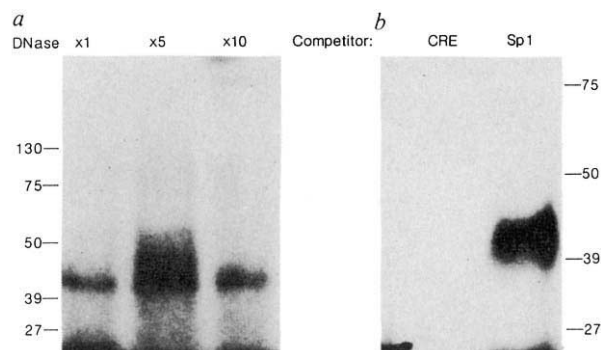


Fig. 3 Cross-linking of CRE-binding protein to somatostatin CRE by ultraviolet light. Cross-linking experiments were performed using a protocol similar to that of ref. 10. A uniformly labelled probe for cross-linking reactions was obtained by replacement synthesis of a DNA fragment containing the somatostatin CRE. After labelling this fragment with [α -³²P]deoxycytosine and bromodeoxyuridine (BUDR), 1 ng (5×10^5 c.p.m.) DNA probe was incubated with 60 μ g unfractionated nuclear extract and 5 μ g competitor poly(dI-dC) DNA. The samples were incubated on ice for 15 min to allow binding of proteins to the DNA. The samples were then irradiated under a Fotodyne ultraviolet lamp (emission wavelength 300 nm, maximum intensity 7,500 μ W cm⁻²) at 25 °C for one hour. CaCl₂ was added to 10 mM and the samples were digested at 37 °C for 30 min with 5–50 U micrococcal nuclease and 2.5–25 μ g DNase I. The samples were then precipitated with 20% trichloroacetic acid and 1 mM deoxycholate, and analysed by electrophoresis through 8% SDS-polyacrylamide gels. The gels were dried and exposed to X-ray film. **a**, Cross-linking reactions were treated with increasing amounts of DNase (1 $\times$ DNase equals 5 units micrococcal nuclease plus 2.5 μ g DNase I). **b**, For competition experiments 25 ng unlabelled DNA competitor was added to samples during the initial binding step. CRE and Sp1 competitors are synthetic oligonucleotides containing binding sites for CREB and Sp1 proteins, respectively. Size markers show M_r in thousands. **Methods.** To prepare DNA probe for ultraviolet cross-linking, $\Delta(-71)$ plasmid was digested with *Hind*III to release a 160-bp fragment containing somatostatin promoter sequences from -71 to +55. After phenol-chloroform extraction and ethanol precipitation, 0.1 pmol *Hind*III-digested $\Delta(-71)$ DNA was digested with 1 U T4 DNA polymerase for 2.5 min. The fill-in reaction was started by adding 250 μ Ci [α -³²P]dCTP (3,000 Ci mmol⁻¹), 50 μ M each dATP, dGTP and BUDR triphosphate. After 15 min, the reaction was terminated by adding EDTA to 3 mM and heating to 65 °C for 10 min. The 160-bp labelled fragment was resolved on a 5% polyacrylamide gel, excised and electroeluted. The incorporation of [³²P]dCTP by the 160-bp fragment was used to estimate the efficiency of replacement synthesis. Approximately 75 nucleotides were removed and replaced on each strand by T4 polymerase. The noncoding strand, therefore, was uniformly labelled over the entire CRE sequence.

prepared, and equal numbers of TCA-precipitable counts from control and forskolin-treated samples were incubated with affinity resin. After washing the resin repeatedly with low salt buffer, affinity-bound proteins were eluted as before in high salt. We observed a 43K phosphoprotein which was specifically retained by the affinity resin. When compared with labelled extracts from untreated cells, the 43K protein appears to be phosphorylated about 3–4 times more in cells treated with forskolin. Several other bands retained by the affinity resin also seem to be induced by forskolin. These may represent other DNA-binding proteins which are also phosphorylated in response to cAMP.

Constantinou *et al.*^{12,13} have proposed that the regulatory subunit of cAMP-protein kinase binds to and stimulates the expression of cAMP responsive genes. The type-2 regulatory subunit appears to possess intrinsic topoisomerase activity¹². By altering the chromatin structure in responsive genes, the regulatory subunit may consequently regulate gene expression. The

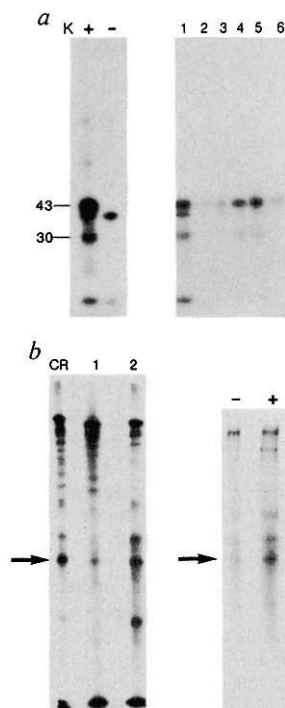


Fig. 4 Phosphorylation of CREB protein *in vitro* and *in vivo*. **a**, Affinity-purified extract (third pass) containing 50 footprint units was incubated with purified catalytic subunit of cAMP-dependent protein kinase in 50 mM potassium orthophosphate (pH 7.2), 10 mM $MgCl_2$, 5 mM NaF. The reaction was initiated by the addition of 2 μ M (20 μ Ci) [γ - 32 P]ATP and incubation was at 30 °C for 5 min. Samples were precipitated with 20% trichloroacetic acid and phosphorylation was assessed by autoradiography following SDS-polyacrylamide gel electrophoresis. Left panel: +, affinity purified extract plus catalytic subunit; -, catalytic subunit only. Size markers show M_r in thousands. Right panel, DNA affinity chromatography of phosphorylated extract. Following incubation with catalytic subunit, purified CREB protein was passed over a 1-ml DNA affinity column (Fig. 2) and 1-ml fractions were collected. Proteins were eluted with increasing concentrations of KCl in buffer Z and precipitated with 20% trichloroacetic acid. Lane 1, flow-through fraction; lane 2, 0.1 M KCl; lane 3, 0.2 M KCl; lanes 4–6, 1.0 M KCl. **b**, Phosphorylation of CREB protein *in vivo*. PC12 cells (5×10^6 per 100-mm dish) were labelled with inorganic 32 P (0.5 mCi ml^{-1}) in phosphate-free medium for 15 h. Cells were then treated with 10 μ M forskolin or ethanol vehicle for one hour. Nuclear extracts (Fig. 1) from the labelled cells were incubated with DNA affinity resin (Fig. 2) and proteins were eluted as above. Left panel: CR, crude nuclear extract from forskolin-treated cells; lane 1, 0.1 M KCl fraction; lane 2, 1.0 M KCl fraction. Labelled fractions were precipitated with 20% TCA, resuspended in sample buffer and analysed on a 10% SDS-polyacrylamide gel. More than 95% of TCA precipitable counts incubated with affinity resin were in the 0.1 M KCl fraction (lane 1). Autoradiographic exposures for each lane were adjusted to allow comparison between fractions. Right panel: 1.0 M KCl fractions from control (–) and forskolin-treated (+) cells. Arrows, 43K phosphoprotein.

M_r of the CRE-binding protein characterized in our report, 43K, differs considerably from that of the regulatory subunit (54K). Genes responsive to cAMP like somatostatin, therefore, appear to be regulated by a nuclear factor distinct from the regulatory subunit.

Lee and coworkers¹⁴ have recently identified a transcription factor, AP-1, which recognizes the sequence -TGACTCA- in the SV40 and human metallothionein II promoters. The AP-1 binding site is homologous to the 'core' motif in the CRE, namely -TGACGTC-, suggesting that CREB and AP-1 proteins may be closely related transcription factors. The characterization of

cDNAs encoding these proteins will reveal the extent to which CREB and AP-1 are homologous.

Our results indicate that cAMP regulates expression of the somatostatin gene through a 43K nuclear protein which binds selectively to the CRE. Cyclic AMP appears to stimulate the phosphorylation of this protein *in vivo*. Furthermore, the purified protein is a substrate for phosphorylation by catalytic subunit of cAMP-dependent protein kinase *in vitro*. Consequently, phosphorylation of CREB *in vivo* may occur directly after activation of catalytic subunit and not by a cascade of protein kinases. CRE-binding activity is not affected by treatment of PC12 cells with forskolin. Cyclic AMP may therefore regulate the transcriptional activity of CREB rather than its binding activity. Alternatively, CREB may interact with another protein which ultimately regulates transcription in response to cAMP. *In vitro* transcription experiments using purified CREB protein will clarify the mechanism by which cAMP regulates gene expression.

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1. Montminy, M. R. *et al.* *J. Neurosci.* **6**, 1171–1176 (1986).
2. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G. & Goodman, R. H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6682–6686 (1986).
3. Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E. & Goodman, H. M. *Nature* **323**, 353–356 (1986).
4. Short, J. M., Wynshaw-Boris, A., Short, H. P. & Hanson, R. W. *J. biol. Chem.* **261**, 9721–9726 (1986).
5. Reichlin, S. *New Engl. J. Med.* **309**, 1495–1501, 1556–1563 (1983).
6. Tapia-Arancibia, L. & Reichlin, S. *Brain Res.* **336**, 67–72 (1985).
7. Shimatsu, A. *et al.* *Endocrinology* **110**, 2113–2117 (1982).
8. Chihara, K., Arimura, A. & Schally, A. *Endocrinology* **104**, 1656–1682 (1979).
9. Kadonaga, J. T. & Tjian, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5889–5893 (1986).
10. Chodosh, L. A., Carthew, R. W. & Sharp, P. A. *Molec. cell. Biol.* **6**, 4723–4733 (1986).
11. Hillel, Z. & Wu, C. W. *Biochemistry* **17**, 2954–2961 (1978).
12. Constantinou, A., Squinto, S. & Jungmann, R. *Cell* **42**, 429–437 (1985).
13. Squinto, S. R., Kelley-Gerraghty, D. C., Kuetel, M. R. & Jungmann, R. A. *J. Cyclic Nucleotide Protein Phosphoryl. Res.* **10**, 65–73 (1985).
14. Lee, W., Haslinger, A., Karin, M. & Tjian, R. *Nature* **325**, 368–372 (1987).
15. Bradford, M. M. *Analyt. Biochem.* **72**, 248–254 (1976).
16. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
17. Morissey, J. H. *Analyt. Biochem.* **117**, 307–313 (1981).

DNA bend direction by phase sensitive detection

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Gel electrophoresis of DNA and protein–DNA complexes has been a key method used in studies of sequence-directed and protein-induced DNA bending^{1,2}. Natural DNA sequences can have protein binding sites adjacent to A-tract bending sites^{3–5}, resulting in the potential for the formation of topologically complex shapes in a localized DNA regulatory domain. An essential first step in deducing the structure and functional significance of such domains is elucidation of the relative direction of bending, which can be determined from the electrophoretic mobilities of isomers having varied helical phasing between the bends. Taking DNA bent around CAP protein as a standard, we conclude that the junction bending model correctly predicts the direction of bending at A_{5–6} tracts in kinetoplast DNA. The overall direction of the bend is towards the minor groove at the centre of the A tract.

We constructed an isomeric set of DNA molecules containing a protein-induced bend next to a piece of DNA that harbours a sequence-directed bend. The phasing between the bends is

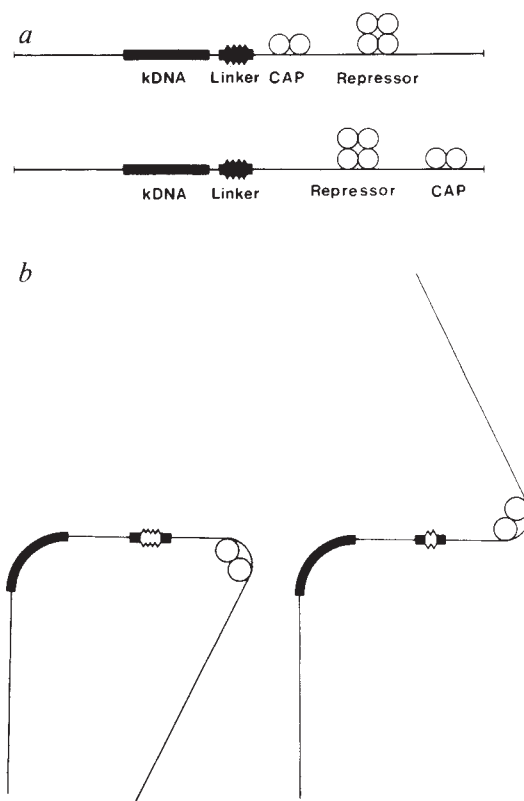


Fig. 1 *a*, Primary structure of the DNA constructs. A piece of DNA of 71 bp (including the *Eco*RI ends) containing the bend sequence from kDNA was inserted into the *Eco*RI site of the polylinker of pGem 2 (Promega). A 156-bp piece of DNA (including the *Bam*HI ends) which contains the *lac* promoter region was then inserted into the *Bam*HI site of the above plasmid. Clones were selected which contained the *lac* promoter region in both orientations (above and below). Linkers which varied in length from 10 to 20 bp were then inserted into this DNA. *b*, Schematic representation of the structure of the DNA molecules containing both kDNA and CAP-induced bends. The kDNA bend is represented as a heavy line, and the CAP molecule is represented as two circles. Left, the *cis* isomer: the two bends are in phase in this molecule. Right, the *trans* isomer: the two bends are out of phase in this molecule.

shifted over one helical turn by the addition of linkers between them, thus altering the end-to-end distance on which gel electrophoretic mobility depends^{2,6,7}. As the orientation of the bends is varied, the mobility of the protein-DNA complexes should increase from a minimum, when the end-to-end distance is short, in the *cis* isomer (Fig. 1*a*) to a maximum in the *trans* isomer.

For the sequence-directed bend, we have chosen a segment from the kinetoplast DNA (kDNA) of the parasite *Leishmania tarentolae*. This DNA contains a regular repeat of the sequence CA₅-6T with ten base-pair (bp) periodicity; the centre of its bend has been determined by electrophoresis of circularly permuted isomers². From the experiments of Koo *et al.*⁸, the bend at A₅-6 tracts lies in the plane defined by the helix axis and the approximate dyad axis through the centre of the A tract, because inversion of the sequence about that dyad does not affect the direction of bending. In the junction bending model favoured by Koo *et al.*⁸, the bend is equivalent to tilt toward the 3' strand at the junctions of the A-T tract with normal B-DNA. Their experiments rule out a dominant bend component in the plane perpendicular to the bending plane in the model; this alternative mode would be equivalent to bending by roll at the junctions, at a 90° angle from the model direction. However, nothing in the experiments rules out bending by tilt toward the 5' strand at each junction, or 180° away from the direction predicted by the model.

As a standard bend we have selected the CAP (catabolite activator protein)-induced DNA bend in the *lac* promoter sequence. It is inferred from chemical protection⁹⁻¹¹ and model building studies¹² that the minor groove of the DNA binding site faces the protein at the approximate dyad axis. Formation of protein-DNA contacts over the large (28-30 bp) binding site required for strong complex formation^{10,13} requires that the DNA be substantially bent toward the protein, by an angle estimated from gel measurements to be between 90° and 180° (ref. 13); there is little net change in DNA linking number¹⁴. Electrostatic calculations and detailed model building predict a sharply bent DNA conformation in contact with a ramp of positive electrostatic potential on three sides of the protein¹⁵. Lacking a true absolute standard, we take as an interim standard

the highly plausible proposed geometry of the CAP-induced bend, whose overall direction is towards the minor groove at the centre of symmetry of the binding site. (However, it should be acknowledged that this direction is not in agreement with apparent kinking of DNA away from the protein inferred from electron microscopic observation of glutaraldehyde-fixed CAP-DNA complexes¹⁶.) We emphasize that our measurements are designed to determine relative bending directions, and the conclusions about absolute direction are dependent on the validity of the standard chosen.

A striking feature of the constructs we made to investigate phasing effects between bends is their enhanced sensitivity to weak intrinsic DNA curvature. We examined *lac* promoter DNA in its two possible orientations with respect to the kDNA locus (Fig. 1*a*). In both cases, even in the absence of protein, there is appreciable variation of gel mobility as the linker length is varied from 10 to 20 bp (Figs 2 and 3). We ascribe this observation to small intrinsic bends in the *lac* promoter fragment¹³, whose influence on mobility is amplified because they provide greater phase-dependent variation in end-to-end distance than would be the case for nearly straight fragments.

We examined binding of *lac* repressor to the set of DNA molecules having the operator site as close as possible to the kDNA bend, which had maximum sensitivity to induced bends. Figure 3*c* shows that the ratio of normalized mobilities for repressor-DNA complex to the corresponding normalized free DNA mobilities yields a nearly straight line. We conclude that the phasing variations of the mobilities of repressor-DNA complexes can be explained on the basis of the bends present in the free DNA, and no new protein-induced bends need be invoked. This result is in agreement with the conclusion of Wu and Crothers² from the circular permutation gel assay.

A lingering uncertainty concerning the CAP-induced bend is the possibility that the bend could be a point of induced flexibility, for example allowing free bending in a defined plane. Such a model, in which there is no bias for bending in one direction in the plane over the other, should show a 5-bp periodicity in the gel mobilities as the bending plane moves into and out of phase with the kDNA bend. As shown by Figs 2*b* and 3*b*, the

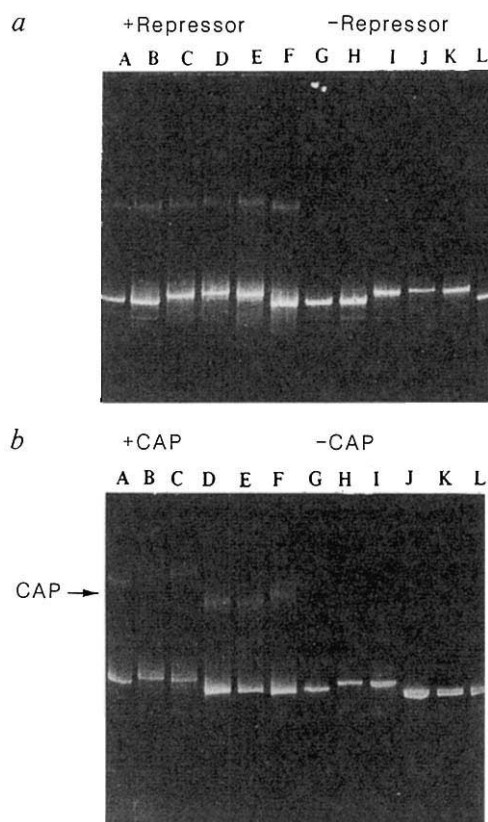


Fig. 2 *a*, Dependence of gel mobility on the phasing between repressor bound to DNA and the kDNA bend. Lanes A-F, repressor-DNA complexes in which the distance between the centre of the repressor site and the centre of the kDNA bend varies in 2-bp increments from 139 bp to 129 bp. Lanes G-L, free DNA corresponding to the DNA in lanes A-F. There remains a small variation of gel mobility with linker length in the observed complex bands. *b*, Dependence of gel mobility on the phasing between CAP bound to DNA and the kDNA bend. Lanes A-F, CAP-DNA complexes in which the distance between the centre of the CAP binding site and the centre of the kDNA bend varies in 2-bp increments from 104-94 bp.

Methods. In *a*, the repressor-DNA complexes were formed by incubating 0.15 μM active repressor (provided by Kathy Matthews) with 0.15 μM DNA in 10 mM NaCl, 50 $\mu\text{g ml}^{-1}$ bovine serum albumin, 10 mM Tris-HCl pH 8.0, 1 mM EDTA in a reaction volume of 20 μl for 20 min at room temperature ($\sim 20^\circ\text{C}$). The complexes were run on a 5% polyacrylamide gel (39:1 acrylamide to bisacrylamide; 89 mM Tris-borate pH 8.2, 2 mM EDTA) for 5 h at 150 V. The gel was prerun for one hour and stained with ethidium bromide. In *b*, the CAP-DNA complexes were formed by incubating 0.15 μM active CAP (provided Abraham Brown) in 25 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 40 μM cAMP for 10 min at room temperature in a 10- μl reaction volume. The complexes were run on a 5% polyacrylamide gel under the same conditions as for the repressor complexes with the exception of the addition of 10 μM cAMP in the running buffer. The gel shown is 30:1 acrylamide to bis-acrylamide. Both 30:1 and 39:1 gels were run, and the numbers which were obtained under these two conditions were identical within the limits of experimental error.

gel mobilities have a period of approximately 10 bp in their dependence on linker length. When the relative mobilities are corrected by dividing by the relative mobility of the free DNA, as for the repressor control (Fig. 3*a*), the variation resembles a harmonic function with 10-bp periodicity. We conclude that the protein-induced bend is indeed directed in space.

The gel mobility of the CAP complex, corrected for variations in the free DNA, reaches a minimum for the 18-bp linker, which must therefore represent the *cis* isomer (Fig. 3*c*). In that molecule a distance of 101.5 bp separates the dyad axis at the centre of the CAP binding site from the central G in the TAGC sequence between the two A_6 tracts at the centre of the kDNA bend. The experimental results of Koo *et al.*⁸ require by symmetry that the effective direction of the kDNA bend be toward either the major or the minor groove at that site. Bending toward the major groove at the centre of the kDNA bend requires a helical screw of ~ 10.6 -10.7 bp per turn to place the two bends 9.5 helical turns apart in the *cis* isomer. The alternative choice of bending toward the minor groove would require helical screw values of 10.1 or 11.3, to yield respectively 10 or 9 helical turns between the bends in the *cis* isomer, well outside the range of reported values⁷⁻²⁰.

This preliminary analysis was confirmed by computing the helix axis trajectory in detail for each isomer, following the procedures described by Levene and Crothers²¹ and using both the junction and 'wedge'^{22,23} bending models as described in Fig. 4. We adjusted the average helical screw between the bends to 10.61-10.62, which is found to yield the *cis* conformation for the 18-bp linker isomer, assuming the bending direction that is predicted by the junction model. We conclude that the kDNA and CAP bend centres lie 9.5 helical turns apart in the *cis* isomer. Separation by an integral number of helical turns (9 or 10), which would be needed if the bending direction were opposite to that predicted by the junction model, was found to require unacceptable helical screw values of 11.2 or 10.2 bp per turn.

We further checked the critical issue of the value of the helical screw by examining cloned constructs containing two CAP sites, which by symmetry must have an integral number of helical

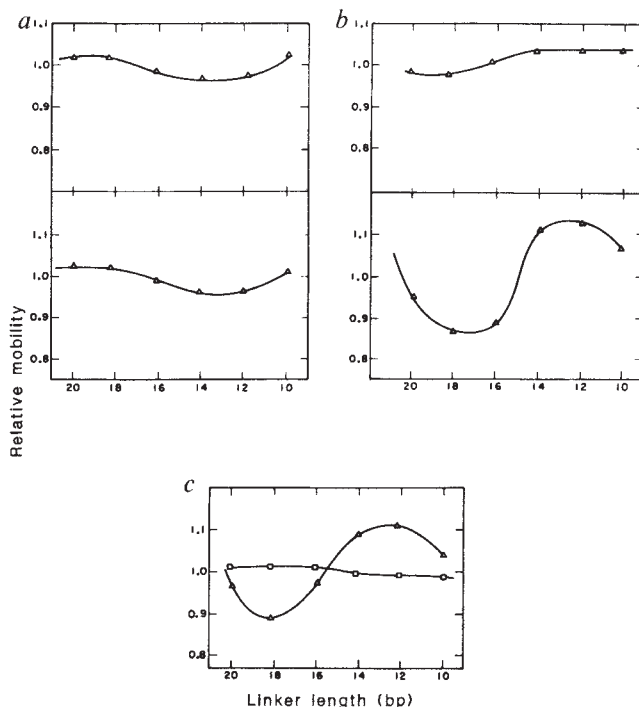
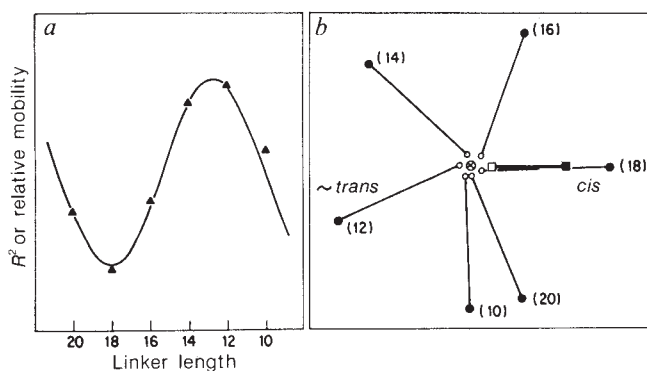


Fig. 3 Relative mobility plotted against linker length. *a*, *b*, Fractional variation in gel mobilities, normalized to the average, for the set of six linker lengths for the repressor-DNA and CAP-DNA complex gels, respectively. In each panel, the top graph is the free DNA, the lower graph for the protein-DNA complex. The fractional variation in gel mobility, normalized to the average for the set of six linker lengths, is very similar for the free DNA and the repressor-DNA complex. *c*, The ratio of the normalized mobilities for the protein-DNA complexes divided by the corresponding normalized free DNA mobility. $\square$, Ratio for repressor; $\triangle$, ratio for CAP.



turns between bend centres in the *cis* isomer. Minimum gel mobility of the doubly occupied complex was found with 84–85 bp between the sites, corresponding to 8 turns and 10.50–10.63 bp per helical turn, strongly reinforcing the conclusion that a value near 10.6 is required in the CAP–kDNA double bend constructs.

The *cis* isomer half-integral phase shift of 9.5 helical turns requires that the overall direction of the bend must be towards the major groove in the centre of the kDNA bend, corresponding to the centre of the TAGGC sequence, because the CAP bend is assumed to be toward the minor groove at the centre of the CAP site. Major groove bending in the centre of TAGGC is the effective bending direction produced by tilt toward the 3' strand at the junctions as defined in the junction bending model⁸. (The junctions average $n \pm 0.25$ helical turn spacing from the bend centre, so bend by tilt at the junctions is geometrically equivalent to bend by roll into the groove at the centre of the bend.) One can also view the bending direction predicted by the junction model as toward the minor groove in the centre of A_{5–6} tracts. Because these centres are located at approximately half integral numbers of helical turns from the centre of the TAGGC sequence, bending into the minor groove at the centre of the A tracts produces an effective bending direction toward the major groove at the TAGGC centre.

Figure 4a shows the calculated variation of the square of the end-to-end distance, compared to the variation in gel mobility, with the two scaled arbitrarily to match in amplitude. Figure 4b shows graphically the computed change in dihedral angle between the two DNA segments that flanked the bends, viewed down the axis of the 'straight' DNA between the bends.

The junction bending model views the intrinsic bend as occurring at the point where the helix axes of the A tract and adjacent B-DNA meet. One can also interpret the bend in terms of the wedge model^{22,23}, in which the bends are associated with angles of tilt and roll between adjacent base pairs. Both models are subject to the symmetry constraints of the experiments of Koo *et al.*⁸, so each can allow bending only in the plane defined by the dyad axis through the centre of the A tract and the helix axis of that segment. Consequently, the models are indistinguishable in the present analysis and our results can be interpreted equally well with either, or with refinements of each (ref. 24; H. S. Koo and D.M.C., manuscript in preparation) designed to interpret the anomalous bending properties of the sequences reported by Hagerman²⁵. However, we note that the junction

Fig. 4 a, Observed gel mobility variation for the CAP–DNA complex (▲), corrected as in Fig. 3c for the mobility variations of the free DNA, compared with the calculated variation of the square of the end-to-end distance, R^2 (solid line) of the series of geometric isomers of varying linker length. The trajectory of the helix axis was calculated using the methods described earlier²⁰ and using the junction bending model. A bend of 11.5° tilt toward the 3' strand was inserted at each end of an A tract. The CAP bend was produced by inserting a roll bend of 25° at half helical turn intervals from positions –10 to +10 relative to the pseudo-dyad axis of the CAP binding site. Bends alternated into the major and minor grooves; the bend at the dyad axis was toward the minor groove. It was found that use of the wedge model^{21,22} for A-tract bends, or concentrating the CAP bend at the central site, had no appreciable effect on the calculated end-to-end distances. The average helix screw of the DNA between the two bends was adjusted to 10.61 to match the optima of the calculated curve to the observed mobility variations. Amplitudes of the observed and calculated mobility variations were scaled by an arbitrary linear factor to match their magnitudes. b, Visualization of the calculated variation of the structure of the linker-dependent geometric isomers. The dihedral angle between the DNA distal to the kDNA bend (5' ■—□ 3') and the DNA distal to the CAP bend (5' ○—● 3') is viewed in a planar projection in a view taken along the segment of DNA connecting the two bends (⊗). The *cis* isomer is found for the 18-bp linker isomer if the average helix screw is adjusted to 10.61 for the DNA between the two bends.

bending model, with its reliance on the structure of poly(dA)·poly(dT) determined by fibre diffraction^{26,27} makes an independent prediction about the absolute direction of bending. It is to the credit of the model that its predicted direction appears to be correct.

DNA retains helix-screw-phase coherence over lengths up to hundreds of base pairs, with the upper limit set primarily by the onset of writhe fluctuations, which greatly increase the total torsional flexibility^{28–31} and therefore dephase distant sites. As a consequence, DNA domains covering hundreds of base pairs can form an organized tertiary structure by a combination of A-tract or other intrinsic bends with bends induced at appropriately phased protein binding sites. It seems likely that these evident structural possibilities are exploited in DNA regulatory regions.

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- Marini, J. C., Levene, S. D., Crothers, D. M. & Englund, P. T. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7664–7668 (1982).
- Wu, H. M. & Crothers, D. M. *Nature* **308**, 509–513 (1984).
- Zahn, K. & Blattner, F. R. *EMBO J.* **4**, 3605–3615 (1985).
- Mukherjee, I. P. & Bastia, D. *Cell* **43**, 189–197 (1985).
- Ryder, K., Silver, S., DeLucia, A. L., Fanning, E. & Tegtmeyer, P. *Cell* **44**, 719–725 (1986).
- Lerman, L. S. & Frisch, H. L. *Biopolymers* **21**, 995–997 (1982).
- Lumpkin, O. J. & Zimm, B. H. *Biopolymers* **21**, 2315–2316 (1982).
- Koo, H.-S., Wu, H.-M. & Crothers, D. M. *Nature* **320**, 501–506 (1986).
- Majors, J. thesis, Harvard Univ. (1977).
- Simpson, R. B. *Nucleic Acids Res.* **8**, 759–766 (1980).
- Schmitz, A. *Nucleic Acids Res.* **9**, 272–292 (1981).
- Weber, I. T. & Steitz, T. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3973–3977 (1984).
- Liu-Johnson, H.-N., Gartenberg, M. R. & Crothers, D. M. *Cell* **47**, 995–1005 (1986).
- Kolb, A. & Buc, H. *Nucleic Acids Res.* **10**, 473–485 (1982).
- Warwick, J., Engelman, B. P. & Steitz, T. A. *Proteins* (submitted).
- Gronenborn, A. M., Nermut, M. V., Eason, P. & Clore, G. M. *J. molec. Biol.* **179**, 751–757 (1984).
- Rhodes, D. & Klug, A. *Nature* **386**, 573–578 (1980).
- Rhodes, D. & Klug, A. *Nature* **292**, 378–380 (1981).
- Wang, J. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 200–203 (1979).
- Peck, L. J. & Wang, J. C. *Nature* **292**, 375–378 (1981).
- Levene, S. D. & Crothers, D. M. *J. Biomolec. Str. Dynam.* **1**, 429–435 (1983).
- Trifonov, E. N. & Sussman, J. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3816–3820 (1980).
- Ulanovsky, L., Bodner, M., Trifonov, E. N. & Choder, M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 862–866 (1986).
- Ulanovsky, L. & Trifonov, E. N. *Nature* (in the press).
- Hagerman, P. J. *Nature* **321**, 449–450 (1986).
- Arnott, S., Chandrasekaran, R., Hall, I. H. & Puigjaner, L. C. *Nucleic Acids Res.* **11**, 4141–4155 (1983).
- Alexeev, D. G., Lipanov, A. A. & Skuratovskii, I. Ya. *Nature* **325**, 831–823 (1987).
- LeBret, M. *Biopolymers* **18**, 1709–1795 (1979).
- Shore, D. & Baldwin, R. L. *J. molec. Biol.* **170**, 983–1007 (1983).
- Horowitz, D. & Wang, J. C. *J. molec. Biol.* **173**, 75–91 (1984).
- Levene, S. L. & Crothers, D. M. *J. molec. Biol.* **189**, 73–83 (1986).

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Confocal microscopy comes of age

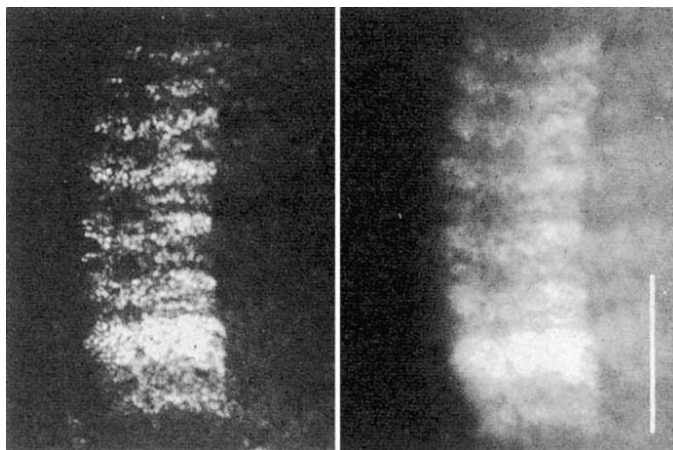
J.G. White and W.B. Amos

Scanning confocal optical microscopy offers increased resolution and superior rejection of out-of-focus signals for epifluorescence imaging of biological material.

In the past 50 years light microscopes have benefited from a succession of new optical developments, including the development of phase contrast, differential interference contrast and fluorescence microscopy techniques. These have made major contributions to our current understanding of biological structures and, although the theoretical foundations of light microscopy were laid down more than 100 years ago by Abbe, new microscopical techniques are still being developed. Computer image processing, for example, now makes it possible to process all of the vast amount of information present in an image, pushing back the limits of what can be seen in light microscopes¹.

The principle behind the scanning confocal microscope was first described in a patent 26 years ago². In a confocal microscope, both illumination and detection are confined to a single point in the specimen. This is achieved by inserting spatial filters (usually pin-holes) into the optical paths of the objective and condenser. A complete image is built up by sequentially scanning all points in the field of view. The technique provides improved resolution and greater rejection of signals from out-of-focus structures than conventional microscopy, where the specimen is bathed in a fairly uniform flux of illumination. While the resolution of a conventional microscope is limited by the point spread function of the objective, in a confocal microscope the total point spread function is the product of the point spread functions of the condenser and objective lenses. If they are equivalent, this can

Fig. 2 *Drosophila* embryo stained with a fluorescein-labelled antibody to the *bithorax* gene product. The bright dots are nuclei of individual cells of the developing nervous system. Confocal image on the left, conventional on the right. Scale bar, 200 μm . Prepared by R. White.



improve resolution by a factor of up to 1.4 (refs 3,4). Confocal imaging also rejects out-of-focus signals, because at any given time, only the point being viewed is illuminated. Out-of-focus or adjacent structures receive little or no illumination and therefore produce a negligible interfering signal.

Given the potential advantages of confocal imaging, it is somewhat surprising that this technique has taken so long to gain general acceptance. The technical difficulties associated with implementing a high speed scanning system, and the requirement to keep the objective and condenser lens in alignment are part of the reason. The use of epi-illumination can solve the alignment problem, because the condenser and objective are one and the same thing in this configuration.

Early confocal imaging systems had a fixed light path and scanned by moving the object^{4,5}. These systems demonstrated the potential of confocal imaging, but did not become widely used, partly because they can take up to 10 seconds to acquire an image. This can be particularly frustrating in biological applications, where a considerable amount of time is often spent in locating a region of interest. Various approaches have been taken to develop a high speed scanning confocal microscope, from the use of a Nipkow disk, similar to that used by Baird in early television systems⁶, to the use of electro-mechanically driven beam deflection systems⁷. High speed scanning confocal imaging systems using beam scanning rather than specimen scanning are now becoming commercially available. These systems use lasers as high brightness illumination sources and computer frame stores to assemble and enhance the

scanned images. Some of these instruments may be attached to a conventional microscope in the same manner as a video camera (Fig. 1), and are almost as easy to use.

The popularity of epifluorescence in biological research stems from the use of fluorochrome-coupled antibodies as highly specific stains, and the development of several indicator fluorochromes for measuring intracellular parameters. Unfortunately, however, the technique is particularly prone to out-of-focus interference, which often gives rise to background haze and flare when viewing intact structures. The traditional solution to this problem has been to cut thin sections of the structure, but this has obvious disadvantages, especially for living material. Confocal imaging almost completely eliminates out-of-focus interference from epifluorescent images (Figs 2 and 3) and allows structures to be sectioned optically. Using this technique, it is possible to reveal structures in biological specimens that are completely obscured when imaged conventionally⁸. It therefore seems likely that this technique will have a considerable impact on studies of cellular structure and organization. Similarly, images such as those shown in Fig. 3 suggest that confocal imaging will have applications in clinical pathology, because the technique yields images from live tissue which are as good as, if not better than, those obtained by the usual method of cutting frozen sections.

Confocal imaging may help to realize the full potential of indicator dyes. These dyes have been developed to determine intracellular parameters, such as pH, membrane potential or free calcium, in living tissue. Free calcium concentrations,

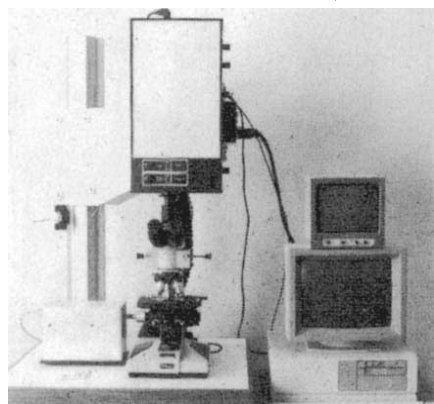


Fig. 1 The MRC/Laserscope confocal microscope attached to the camera port of a conventional microscope. The frame store and control circuitry are housed in the computer on the right hand side. The small box on the left houses the motorized focusing control unit.

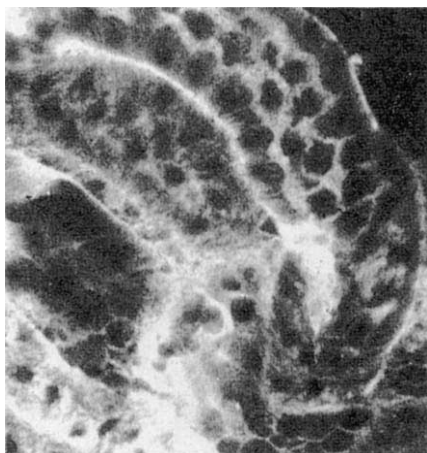


Fig. 3 Cut surface of a fresh unfixed mouse kidney stained intravitaly with a potential sensitive styryl dye. The confocal image (top) shows a kidney tubule, also recognizable in the conventional epifluorescence image of the same region (bottom). Scale bar, 200 μ m. Prepared by A. Fine.

for example, have been obtained by measuring the ratio of fluorescence intensity at two different emission or excitation wavelengths of certain specially developed fluorochromes⁹. With conventional imaging, intensity measurements include signal contributions from regions outside the plane of focus, making it difficult to estimate the true signal from the region of interest. Confocal imaging should virtually eliminate this problem, and will facilitate the direct visualization of physiological parameters in living tissue. □

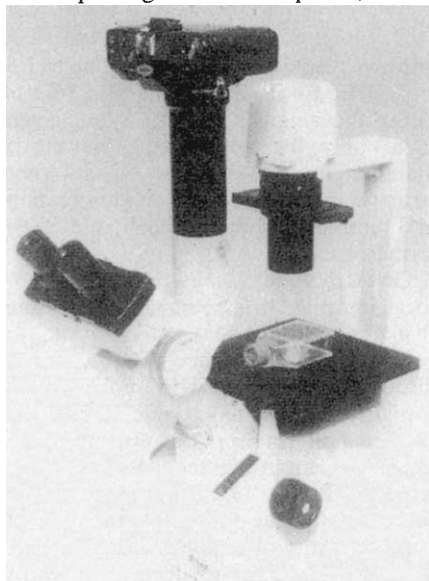
John White and Brad Amos are at the Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge CB2 2QH, UK. For more information, fill in reader service number 100.

1. Agard, D.A. & Sedat, J.W. *Nature* **302**, 676-681 (1983).
2. Minsky, M. U.S. Patent no. 3013467.
3. Wilson, T. & Sheppard, C.J.R. *Theory and Practice of Scanning Optical Microscopy*, (Academic, New York, 1984).
4. Brakenhoff, G.J., Blom, P. & Barends, P.J.J. *Microsc.* **117**, 219-232 (1979).
5. Wijneandts van Resant, R.W. *et al. J. Microsc.* **138**, 29-34 (1985).
6. Petráň, M., Hadravský, J., Benes, J., Kucera, R. & Boyd, A. *Proc. R. Microsc. Soc.* **20**, 125-139 (1985).
7. Carlsson, K. *et al. Optics Lett.* **10**, 53-55 (1985).
8. White, J.G., Amos, W.B. & Fordham, M. *J. Cell Biol.* (in the press).
9. Tsien, R.Y. & Poenie, M. *Trends biochem. Sci.* **11**, 450-455 (1986).

Focusing on microscopy

Tools for a wide variety of microscopical techniques are described this week, including a high-power SEM, a laser scanning microscope, and image processing systems to suit a range of applications.

A PHOTOMICROSCOPE designed for routine cell and tissue culture work is available from Nikon (*Reader Service No. 101*). The TMS-F inverted microscope is fitted with a photographic tube that accepts a conventional 35 mm camera or a closed circuit television camera. Using closed circuit TV equipment, results can be monitored from a remote location, or be processed through image analysis. The £1,800 (UK) microscope has sensitive 8.8 mm focusing, and Nikon says the 20 W halogen lamp provides bright images even with the $\times 40$ phase constant mode. Options include an attachable mechanical stage for scanning and inspecting 96-well test plates, and a



Nikon's inverted photomicroscope for tissue work. holder for 60-well Teraski plates, Petri dishes and slide glasses. The illuminator stand detaches for inspecting large specimens, when an external illumination source such as a flood lamp can be used.

A new trinocular stereoscopic microscope has joined the ranks of Gallenkamp's microscope lineup (*Reader Service No. 102*). The model 330 is a zoom microscope with a vertical phototube, through which cameras can be mounted using a series of adaptors. Gallenkamp's £970 (UK) microscope is available on either a bench stand or a large universal arm to accommodate large specimens, and can be illuminated with either its own built-in sources or fibre optic illuminators.

From the engineers at Zeiss comes a laser scanning microscope with a zoom capability of up to $\times 8,000$ (*Reader Service*



Zeiss is just starting to take orders now. No. 103). The instrument combines a microscope and conventional imaging facilities with a laser scanning mode to achieve high image contrast and optical performance. The system can pick up low light signals including fluorescence, and can be focused using confocal imaging for depth discrimination. The laser source can also be used to interact with the specimen for excitation and quenching of fluorescence, and for the production of electrical currents.

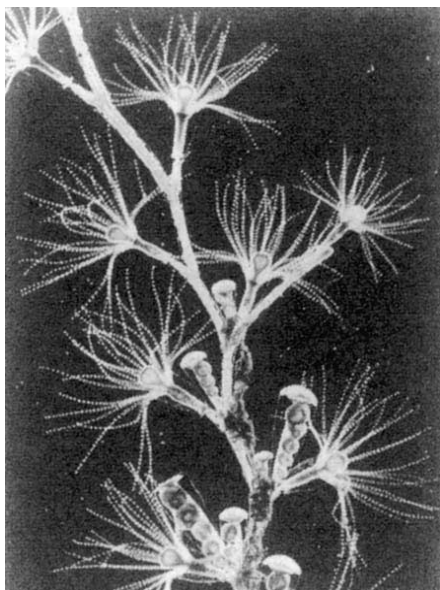
Image analysis

Cambridge Instruments has an image analysis system, the Quantimet 970, that can perform Petri dish bioassays of antibiotic potency (*Reader Service No. 104*). The Quantimet system uses an integrated image analysis computer and Cambridge's bioassay software to yield results less prone to error than data collected with conventional manual bioassay methods. The system works by measuring zones of inhibition at a rate of 2 to 3 seconds per dish, and comparing them to a set of standards. Software for other biological applications is also available, including packages for neurological studies, bone

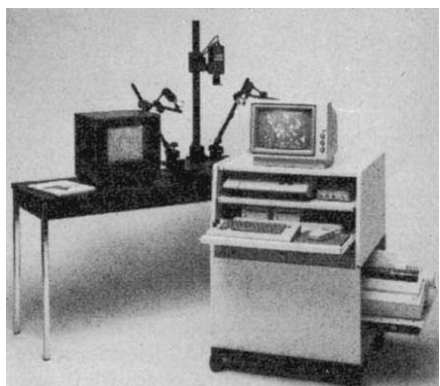


For assaying potency of antibiotics, and more. morphometry analyses, nuclear/cytoplasmic ratios, colony and cell counting, gel electrophoresis and chromosome analysis.

The ImageWorks colour display system from Dapple Systems is designed to



This feathery image of the hydroid polyp *Gonothyrea lovenii*, captured on film by Robert Brons of the Netherlands, was the grand prize winner in the 1986 Polaroid International Instant Photomicrography Competition. The competition accepts entries from all over the world in four categories — black and white micrography, colour micrography, electron micrography, and 35 mm slide micrography — as long as they were created using Polaroid instant films. Eight cash prizes, ranging from \$100 (US) to \$1,000 (US) are awarded for each category. The grand prize winner is selected from the first-place category winners, and receives an eight-day vacation for two to Honolulu, Hawaii or Paris, France. There is still time to get entries in for this year's competition: the deadline is 24 August 1987.



The ImageWorks system colours in microscope images.

pseudo-colour process grey-scale images captured from the video output of optical microscopes, macroviewers or electron microscopes (*Reader Service No. 105*). The \$35,000 (US) ImageWorks system selects from four preset 64-colour tables to enhance the grey-scale image and accent detail. Features may be coded with a colour range to indicate maximum and minimum values for parameters under study, such as area or perimeter. Slow scan im-

ages or X-ray elemental maps acquired from an SEM may also be processed.

Digithurst has souped up its MicroScale IIR image processing system with the addition of an **enhanced graphics adaptor** (EGA) card, so that it now displays pseudo-colour images on an EGA screen (*Reader Service No. 106*). The assignment of colours is controlled by colour look-up tables that can be defined by the user, to match the original image as closely as possible. The system's mouse-driven cursor can be used to measure intensities and distances on both the monochrome and EGA screens. Lower and upper thresholds can also be selected from the colour bands on the EGA display using the cursor. For studies of specific areas, users can specify rectangular regions of interest or windows to exclude sections of



An EGA card expands the MicroScale IIR's abilities.

the image that are not being studied. The Digithurst Microscale IIR system, including EGA, costs £6,450 (UK).

Image Data Systems has an **image processing system tailored for the neuroscientist**, that performs autoradiographic tissue slice analysis (*Reader Service No. 107*). The Bio Imaging Computer System (BICS) consists of an IBM AT-compatible computer, CCD camera and mast, light table, mouse, colour and monochrome displays, Brain Slice Analysis Software (BSAS), printer and desk, and costs under \$40,000 (US). Image Data says the system gathers information on specific chemical binding sites by digitizing the film sample and calculating appropriate statistics. Subjects under analysis are displayed in colours assigned to density levels on a high resolution display. Areas of interest for data gathering and point density measurements are outlined with the mouse. The BICS system provides image enhancement with functions for smoothing, edge detection, contrast stretching, and three-dimensional relief in monochrome or colour with various selectable resolutions. Custom formatted reports can be generated using the system, and the database is

compatible with SPSS, BMDP, Lotus 1-2-3, Symphony, dBase III, Reflex, and other software for statistical comparison and analysis.

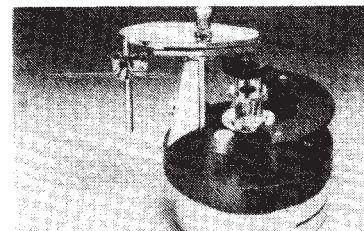
Microscience Inc. has added a new **morphometry module** for PC-based image processing to its library of ImageMeasure software (*Reader Service No. 108*). The IM2500-Morphometry software is IBM-compatible, and was designed to measure the main dimensional attributes of objects. It performs counts, lengths, widths, perimeters, and areas, and measures number densities, positional information, angular orientations, and shape parameter. Measurements are given in units and as a percentage of reference values, and as many variables as needed for a particular data set can be measured. Each measurement can be classified according to type, and the class description includes "macros" that tell the system what type of measurement to perform. Built-in analysis routines compute statistics for each classification and variable in the data file. A complete system, including hardware, sells for less than \$10,000 (US).

Electron microscopy

Jeol's scanning electron microscope, the T330, focuses on ease of operation

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(Reader Service No. 109). Jeol says its SEM is full of automatic controls including astigmatism correction, contrast and brightness, and magnetic compensation with parameter changes. The T330 also monitors and accommodates changes in focus due to adjustments in specimen height, and has an automatic facility to search the image to sharpen detail at the touch of the focus button. Specifications include 5 nm resolution, magnifications ranging from $\times 15$ to $\times 200,000$, and accelerating voltage from 0.5 to 30 kV. For viewing uncoated surfaces, a low voltage addition is offered, that goes from 0.5 kV to 3 kV in 100-V steps. The stage area of Jeol's SEM can hold a 5-inch specimen, and a cryo unit can be added to keep tissue sections frozen during inspection. The character display keyboard allows the user to record specimen numbers, conditions and other data on the micrographs; parameters such as accelerating voltage, magnification and micron value are recorded automatically when the micrograph is taken.

Philips has a scanning/transmission electron microscope with operating voltages of up to 300 kV, geared for high resolution imaging and analysis (Reader Service No.

110). The CM30 electron microscope is packed with imaging and analytical features: high resolution TEM imaging, small TEM probes, CBED with a large diffraction projection angle, and STEM imaging in bright- and dark-field modes. X-ray analysis, EELS detection and hybrid TEM scanning can also be performed. Philips says the CM30 combines analytical transmission microscopy with high resolution structure imaging down to a point resolution of 0.23 nm. The instrument is ergonomically designed: a user can access all of the controls required for TEM, STEM or hybrid scanning modes from only two operating surfaces.

Micro-tools

The Hoffman Modulation Contrast System from Modulation Optics can be used to produce a high-contrast, high-resolution image, that appears to be three-dimensional (Reader Service No. 111).



All you need for Hoffman Modulation Contrast work.

The components are available for research, clinical, student and inverted microscopes, and produce no halo artefact, according to the company. The \$4,000 (US) system, including turret condenser and a full set of planachromat objectives, can image through glass or plastic, and can be used with epifluorescence and bright-field microscopy.

With the microscopic high speed video system from International IMC Ltd, it is possible to slow down the fast movements of cilia, spermatozoa and flagella for study (Reader Service No. 113). The £33,000 (UK) NAC MHS-200 system records 200 pictures a second, and uses VHS video tape. The cold light source gives exposure times of less than 1/70,000 sec. and record-



Speedy spermatozoa are no match for International IMC Ltd's high speed video system.

ings can be made at brightness levels as low as 30 lux, according to the company. The system records over half an hour of movements, and automatically records and displays experiment numbers and time information in the replayed sequences. It can be used with any microscope, erect or inverted, and pictures can be played back in normal, slow, still, reverse or single frame advance.

For extremely low light level imaging in fluorescence and luminescence applications, Photonic Microscopy has an ultra-sensitive video camera, the C2400 VIM (Reader Service No. 112). The \$33,000–36,000 (US) camera uses a two-stage MCP image intensifier, and provides low noise and a dynamic range extending from visible to single photon levels, according to Photonic. Image pickup tubes can be chosen to allow the camera to be configured for conventional analogue or photon counting imaging, and the system is available with a spectral response range of either 280–650 nm or 280–850 nm. Standard features include overload protection circuitry and bias lighting. Peltier cooling, shading correction, gamma correction, polarity inversion and external synchronization control are optional.

For keeping specimens under study at the right temperature, Sensortek offers a thermal stage that can maintain temperatures as high as 100°C (Reader Service No. 114). The \$2,600 (US) stage can be used



Sensortek's thermal stage keeps cultures and other samples out of the cold.

with most microscopes and fits onto the mechanical stage, so it can be moved with the regular mechanical stage controls. The stage has a hole for use with transmitted light. The temperature is adjusted using controls on the thermoelectric device, and Sensortek says it is accurate to 0.1°C over a temperature range of between –20°C and +100°C. The unit requires a 110 V or 220 V a.c. current, and must be hooked up to a trickle of water. □

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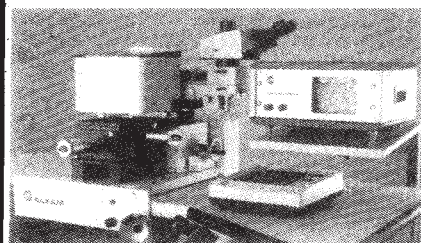
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